THE STRUCTURE AND MODE OF GROWTH

OF BACTERIAL COLONIES.

Thesis submitted for the Degree of Ph. D.

CONTENTS.

The greater part of the data included in this thesis is embodied in two papers by the author: "The Structure of Rough and Smooth Colonies", published in the Journal of Pathology and Bacteriology, vol.xlvii, No.2, pp.223-229, 1938; and also "The Mode of Growth of Bacterial Colonies" in the same journal, vol.xlviii, No.2, pp.427-435, 1939.

INTRODUCTION.

The bacteria were among the first organisms to be studied by the methods of microscopy, and in con-:sequence of the motility which they so frequently exhibited, were at first regarded as animals by the naturalists of the day; animals being believed, at that time, to possess a monopoly of this quality. When, with the advance of biological knowledge, motility was observed to be commonly shown by many unicellular forms of plant life, and by creatures which appeared to occupy an intermediate position between plants and animals, attempts were made to discover further criteria of identification by which the place of bacteria in the world of living things might be more accurately determined. Accordingly Cohn (1853) studied their growth and mode of life, and on the return swing of the pendulum, placed them among the green plants, within the Mycophyceae a sub-group of the algae. This classification was not allowed to stand for long undisturbed however, for within four years von Nägeli (1857) stated that he considered/

considered the grounds for classifying the bacteria as plants to be insufficient, and suggested that they belonged rightly to the group of Fungi. At the same time he applied to them the name of Schizomycetes, by which they are known to this day.

These variations of opinion cannot be regarded as surprising, in view of the fact that the taxonomic position of the bacteria still remains a matter of considerable doubt. We cannot overlook either the obvious resemblances between their mode of life and that of the higher fungi, or the forms which exist, apparently intermediate between the two, the Actino-:mycetes or fungus-like bacteria, and the yeasts, which closely resemble bacteria although classified as fungi. Yet on the other side of the picture there exist the autotrophic bacteria, which share with the green plants the ability to find their sources of energy and nutritive substance in complete indepen-:dence of previously elaborated organic matter.
The difficulty of the problem is in no way lessened by the fact that these autotrophic forms, the only ones capable of existence in an otherwise lifeless world, are by no means the least complex of the bacteria from a structural viewpoint. The tendency to regard the heterotrophic bacteria as the most primitive/

primitive form of living cell has accordingly been modified, both in consequence of the inevitable con:clusion that all living things must ultimately be descended from autotrophic ancestors, and also of the increasing complexity which, as our knowledge advances, is shown to exist in those species which were pre:viously regarded as simple, both in their structure and mode of life.

The earlier workers who attempted classifications on Linnean principles within the group of bacteria, possessed very few criteria by which the qualities of these organisms could be determined. As a group they were observed to multiply mainly, if not solely by transverse fission, and it was observed that, as in the case of more complex organisms, in some forms the daughter cells parted completely on division, while in others they remained attached to one another, forming long threads. They were studied mainly in infusions of hay or other organic matter, and it is unlikely that any of the infusions examined by these workers even approximated to pure cultures. In consequence of this, very few species could be distinguished, those that were described (entirely on morphological grounds) appearing to represent a heterogeneous collection of what we now recognise as different types of/

of bacteria. The majority of writers placed all the modern group of Eubacteriales in a family Vibriones, within the Infusoria, and comprising a small number of genera including the genus Bacterium in which bacillary and filamentous forms were indifferently grouped. The definition of this genus given by Ehrenberg in 1838 is - "animal e familia Vibrioniorum divisione spontanea in catenam filiformen rigidulam abiens"; while Dujardin in 1841 wrote similarly - "Corps filiforme roide devenant plus ou moins distinctement articule .....".

These definitions while including the chained and threaded forms of the modern genera Bacterium and Bacillus, can also be taken to include such distantly related forms as the Streptothrices and Actinomyces. It is doubtful whether these writers intended to include such forms, or were indeed aware of their existence, but in a work of slightly later date we find what appears to be the first undeniable reference to the existence of species which are capable of growing both in the form of individual bacilli and of uninterrupted threads: this is to be found in the classification of bacterial species given by Perty in 1852, in which he included a genus Metallacter com:prising a single species, M. bacillus which was distinguished/

distinguished by the fact that it grew normally in the form of individual bacilli but might upon occasion extend by growth into long, non-motile threads which he likened to the alga Hygrocrocis. This observation appears to have been regarded with suspicion by subse:quent workers, but it is hoped to show below that it refers, in all probability, to what is now a well-recognised type of bacterial dissociation.

When systematic bacteriology had reached a more advanced stage, the attention of numerous workers was drawn to the distinctive appearance of the colony of the anthrax bacillus, which has been variously des:cribed as resembling a lock of hair or the snake-wreathed head of the Gorgon Medusa. The large size of this organism rendered it easy to discern that the wreathed appearance of the colony was due to continuous chains or threads of bacilli attached end to end, weaving in and out about the periphery. This interest was renewed by Pasteur's production of variants in this species by growth under adverse conditions. The minute structure of the colonies of these variants, and of the original "Medusa head" colony was described by Preisz (1904, 1911) and later by Nungester (1929) and Nungester and Jung (1931-32) and similar studies were made on the closely related B. subtilis by Soule/

Soule (1928). One of the commonest types of variant produced a smooth, mucoid colony which differed from the previously described form in being composed of bacilli, not in chains but lying separately, in pairs or in small bundles. These variants differed also from those composing the "Medusa head" colonies, in being of reduced virulence and possessing the power, absent in the virulent form, of producing a uniform turbidity in broth culture or a stable suspension in saline solution.

The study of colonial form acquired special importance from the studies of Arkwright (1919-20, 1921, 1926, 1927a and b, 1929 and 1930; Arkwright and Goyle, 1924) in relation to bacterial variation. He described the occurrence of rough or "R" colonies in cultures of various members of the coli-typhoid group whose normal colonial form was smooth or "S". These variants, like those of the anthrax bacillus, showed correlated changes in pathogenicity and antigenic structure, while the ability to form a stable suspen:sion in saline solution was found to be characteristic of "S" but not of "R" forms. At about the same time De Kruif (1921) described the dissociation of an avirulent variant from a virulent ~~organisms~~ organism of the Pasteurella/

Pasteurella septicaemia group, the virulent form pro:ducing a stable suspension in saline, while the avirulent variant was spontaneously agglutinable.

In the light of these newer discoveries the observations of Pasteur and Preisz have been inter:preted as indicating that the normal phase of the anthrax bacillus is R, and that the variation to an S form is homologous with the S → R variation of the coli-typhoid organisms. Curiously enough, the obvious corollary that the minute structure of an R colony of a coli-typhoid bacillus might resemble that of the anthrax bacillus has received little attention. The occurrence in R cultures of long,, thread-like forms was frequently observed (Hadley, 1927, 1937; Arkwright, 1930), but the examination of such cultures by means of film preparations led these workers to describe certain R colonies as possessing a "tangled mycelium", and although "Medusa head" colonies were observed in various bacterial species (Arkwright, 1930) they did not receive special attention as they frequently possessed the antigenic structure of an S form. In 1938 however, Hoogerheide described rough colonial variants of Clostridium histolyticum in which the bacilli grew in filamentous form. These variants also showed changes in their biochemical behaviour, so that/

that they closely resembled Clostridium sporogenes in all respects. This has led Hoogerheide to believe that a genetic relationship exists between these two species.

The mode of growth of bacteria also received some attention. As long ago as 1898 Serkowsky showed that bacterial threads grew all along their length, and not merely at the ends in the manner of the majority of higher organisms, and a fairly comprehensive survey of the growth of various bacterial species was made by Graham-Smith (1910), his findings being very generally accepted among bacteriologists. He described division as occurring with "snapping", "slipping" and various other modes of separation, according to the type of bacterial species. The author will attempt to show that these findings were greatly influenced by tech-:nique.

When attention and interest had been drawn to the appearance of rough and smooth colonies, various workers (Nutt, 1927; Seal, 1937) investigated the growth of these colonial forms, directing their investigations mainly to a study of the methods of division of the different types of organism. In both cases, working respectively with dysentery bacilli and cholera vibrios, the conclusions reached by these workers were that the

S/

S organisms slipped past one another on division, while the R strains tended to divide with a snapping movement, in consequence of their much firmer attach:ment, producing a colony in which the bacilli at the edge were arranged in "an irregular and haphazard manner". All these observations were made upon organisms growing in solid medium under cover-slips, and were perfectly valid for growth in these circum:stances but some of the conclusions which were drawn from them were misleading. An exceedingly detailed account of the development of rough and smooth forms of <u>Bacillus lactis niger</u> on the surface of solid medium, from a single organism to a small group, was given by Lasseur <u>et al</u>. (1937), in which the smooth bacilli were shown to separate completely on division, while the rough forms grew as an uninterrupted thread. Some observations were also given on the structure of the edges of grown colonies of this and other species, the bacilli at the edges of the rough colonies being described as lying in parallel chains. The apparent reason why the development of the colonies between these points was not referred to, was that all the colonies and growing organisms were examined unstained and <u>in situ</u>, under which conditions the internal structure of a colony is difficult to determine.

Some/

Some mention should be made of the work of Pijper (1938), who, using a special technique of dark-ground illumination, examined the structure of the floccules produced in the phenomen of "somatic" agglutination of coli-typhoid organisms. In these masses the bacteria were found to be adhering to one another by the ends only; the general appearance being that of tangled chains rather than compact masses. This is particu-:larly interesting in view of the fact that S $\rightarrow$ R variation is a very frequent after-effect of such agglutination.

Despite the enormous amount which has been written on the subject, very little is known of the mechanism controlling division in the bacteria. Discrete nuclei have been described in many species (Vejdowsky, 1900, 1904; Dobell, 1911; Douglas and Distaso, 1912; Stoughton, 1929), and while some of these organisms are undeniable bacteria, there are others which have received considerable criticism in this respect, and are considered by certain writers to resemble yeasts or lower fungi. It is unfortunate, in this connection, that the majority of these "bacteria" were isolated from such sites as the alimentary canals of invertebrates, which are known to/

to be parasitised by a vast number of different species of micro-organisms of highly aberrant form. It is also unfortunate that in very few cases was any attempt made to cultivate them artificially, while some were observed only in tissue sections. The same criticism applies to much of the work published by Schaudinn (1902, 1903) and Dobell (1908, 1909a, b,c) which describes the occurrence in such bacteria, of diffuse nuclear material, in a granular or reticular form. This variety of structure was also reported however, by Guilliermond (1906, 1908, 1910) in members of the family of Bacillaceae, and in other bacteria of unquestionable authenticity. These workers also described discrete nuclei of the appearance of spiral threads in bacillary forms, while Swellengrebel (1907) and Dobell (1909c) described similar organisms in which the nucleus was capable of adopting either a discrete or a spiral appearance at different times.

The subject was summed up in an exceedingly exhaustive monograph by Dobell (1911), who advanced the theory that the diffuse nucleus of those bacterial forms which he found in the intestines of lower verte-:brates was analagous to that of the Opalina group of ciliates, which are found in similar sites, and that the condition in both groups was a degenerative change due/

due to long-continued parasitism.

A later summary by Guilliermond (1921) favours the view that the majority of bacteria possess a diffuse nucleus, but that the existence of typical nuclei in the group has yet to be conclusively proved. He suggests that some of the structures of this nature described, actually represent the septa produced by certain bacteria in the process of division, which stain deeply with the nuclear dyes. It is interesting to note that the figures of Schaudinn also show this deep staining of the divisional septum, although he does not remark on the fact in his text. A still later monograph by Gotschlich (1927) however, claims the existence of true nuclei in many bacterial species.

Very little attempt has been made to correlate these characters with the vital processes or life-history of the bacteria. Schaudinn described the life-cycle of a large organism isolated from the gut of cockroaches, in which the nuclear material appeared to be diffuse, and which formed a well-marked septum when dividing, but most of his attention was confined to the phenomenon of sporulation. Guilliermond described similar mechanisms in members of the Bacillaceae. The organism observed by Douglas and Distaso, which was a small, Gram-negative cocco-bacillus/

bacillus isolated from the respiratory passages in cases of pulmonary infection in human beings, possessed a discrete central body which completed its division slightly in advance of the cytoplasm of the cell, and became diffuse or vanished altogether in the case of involution forms found in ageing cultures.

The division of the cells does not otherwise appear to have been given much attention; most of the writers referred to described it as "simple", but this term, as applied to those forms producing septa or similar structures, as well as to those whose separa:tion is almost amoeboid in character, is not parti:cularly significant, and probably refers mainly to the absence of any observable process resembling mitosis, even in the nucleated species.

For many years great attention has been paid to the macroscopic appearance of bacterial colonies, many of which are distinctive in appearance, and may be particularly so when growing upon specially designed, differential media. In these circumstances the colonial appearance of certain bacterial species has come to be regarded as of primary importance in iden:tification, and has even served to differentiate minor groups within species. Although growth upon artificial medium has been recognised as foreign to the nature of most bacteria, it has been assumed that the/

the appearance of such cultures is a constant function of the species in question, and not subject to fortuitous alteration. Very few attempts have accordingly been made to determine the reasons for these appearances, although it has frequently been pointed out that the subject is one in which further knowledge is required.

It had frequently been observed in this laboratory, that whereas the appearance of certain bacillary organisms in stained films made from old laboratory cultures was usually quite typical of the species in question, impression preparations made from the same cultures frequently showed large numbers of filamentous forms. This suggested the possibilities of a systematic survey of bacterial colonies by this means, and the correlation of minute structure with other characters, particularly those associated with S $\rightarrow$ R variation. This has been made upon the following organisms: <u>Bacillus anthracis</u>; <u>Bacillus anthracoides</u>; <u>Clostridium welchii</u>; <u>Corynebacterium diphtheriae</u>; <u>Diplococcus pneumoniae</u>; <u>Eberthella typhosa</u>; <u>Escherichia coli</u>; <u>Klebsiella pneumoniae</u>; <u>Mycobacterium phlei</u>; <u>Shigella dysenteriae</u>; <u>Shigella paradysenteriae</u>; <u>Shigella paradysenteriae var. sonnei</u>; <u>Streptococcus haemolyticus</u>; <u>Streptococcus viridans</u>; <u>Vibrio cholerae</u>; and/

and has been extended by observations made upon growing organisms and colonies of various types. It is hoped to show below, something of the structure of these colonies, and the nature and mode of action of the forces which determine it, as well as the bearing which these observations may have upon the relation-:ships and inter-relationships of bacteria.

TECHNIQUE.

The majority of observations were made upon impression preparations from growths on any of the ordinary solid laboratory media, horse blood agar giving, on the whole, rather better results than most other types, although with sufficient care any medium may be used. The advantage of the blood agar appeared to be in the adhesive proteins which held the medium firmly to the coverslip; neither serum agar, however, nor Loeffler's nor MacLeod's medium appeared to possess this property to a greater extent than ordinary agar, so other factors may have exerted some influence.

The plates used were perfectly dry on the surface, and as free as possible from irregularities. Inocu:lation was performed with a glass rod, as a wire loop was found to be liable to make scratches on the surface of the medium. Preparations were made of colonies ranging in age from 6 to 48 hours, the size being the most important consideration. Where colonies of small size were required, it was possible to inoculate the plates much more heavily than would be the case if it were intended to produce discrete colonies after a protracted period of growth. This was advantageous when it enabled preparations to be made comprising a large/

large number of single colonies on one section of medium. Colonies of much greater size could be utilised where these were tough and resistant to distortion or multiplication, as in the case of the anthrax bacillus, and to a less extent, of any rough form, than could be made use of in the case of those of the much softer smooth forms, preparations of which were exceedingly difficult to make under any circum-:stances.

The grown plates were first searched with a lens or the low power of the microscope, to discover colonies suitable for study, the position of which, if they were too small to be readily detectable by the naked eye, could be marked on the back of the plate with a grease pencil. The section of medium bearing the colonies was then cut out with a sharp knife, and transferred as quickly as possible on to a coverslip, one edge of the face of the medium being first pressed on to the glass, and the remainder allowed to attach itself by surface attraction, starting from this edge, thus reducing to a minimum the risk of injuring the colonies or trapping bubbles of air between the medium and the coverslip.

The coverslip and medium attached were then immersed in Bouin's fixative until the medium was blanched/

blanched throughout, usually for about two hours. The medium was then peeled away from the coverslip by inserting the point of a knife or needle between the two surfaces, while holding the medium gently in place with the fingers, to avoid any trace of lateral move-:ment. When the medium was removed the colonies remained attached to the coverslip. The fixative was then washed out by leaving the preparation soaking for some hours in water. This was found to be particu-:larly necessary in cases where stains were used which form an insoluble precipitate with picric acid, e.g. methylene blue, but in the case of large colonies with thick centres it was found that the picric acid itself was a very useful dye, and was left intact. When other stains were used, the preparation was stained in the ordinary way, the most useful stains being basic fuchsin and gentian violet. The former, although giving excellent results when photographed, was inferior to the latter for purposes of examination by eye.

From this point onwards, the preparations could either be dehydrated, cleared in xylol and then mounted in balsam after the manner of sections, but it was found that careful drying of the stained preparation, and/

and direct mounting in balsam, gave much better results, except in the case of large, smooth colonies, which were apt to break up under this treatment.

Direct Observations.

Studies were also made of living bacteria and colonies. Two methods were employed for this purpose; the first, consisting simply of observations of growing colonies on ordinary agar plates, in a microscope incubator, under the 16 mm. objective of the microscope, was used mainly as a check on those made by means of impression preparations, and to observe the preliminary stages in colony formation. Under these circumstances organisms could be discerned more readily by the 16 mm. than by any other power of the microscope, those of the size of coliform bacilli being readily observable at this magnification. Where the plates were poured of suitable thickness, the microscope was focussed through the bottom of the Petri dish, which was incubated in its normal, inverted position. This avoided the necessity of opening the plates for long periods of time, with the consequent danger of drying and con-:tamination.

The other method was used when it was desired to study the modes of division of the bacteria themselves, under/

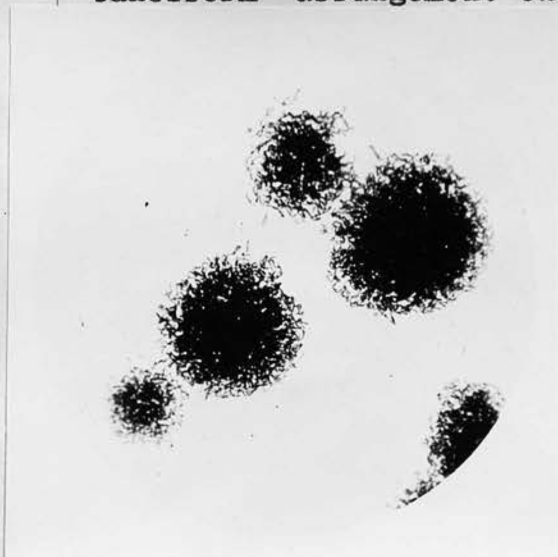
under as high a power as possible. For this purpose they were grown under coverslips, on the surface of semi-solid agar in watch-glasses. Semi-solid agar (0.3%) was used in preference to ordinary agar, in order to interfere as little as possible with the natural growth of the bacteria, but it should be noted that the observations made under these conditions were of their internal mechanisms, which should not be subject to much influence from external forces.

OBSERVATIONS MADE.

SECTION I.

Structure of Colonies.

The first preparations were made from five strains of C. diphtheriae (two gravis, one intermediate, two mitis strains). The medium used was blood agar, and the cultures were usually incubated for six to ten hours, yielding colonies of about 0.5 mm. to 0.25 mm. in diameter. These at first consisted of masses of individual bacilli, lying in their characteristic "cuneiform" arrangement one to another (Plate 1).



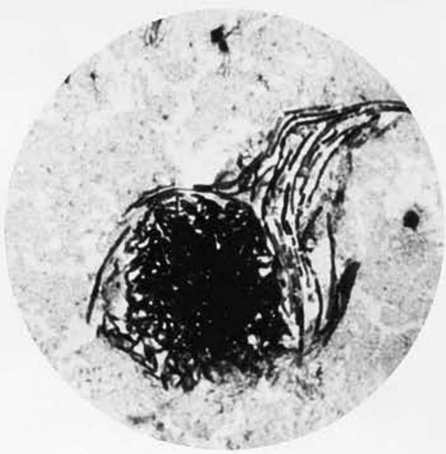
C. diphtheriae (mitis)

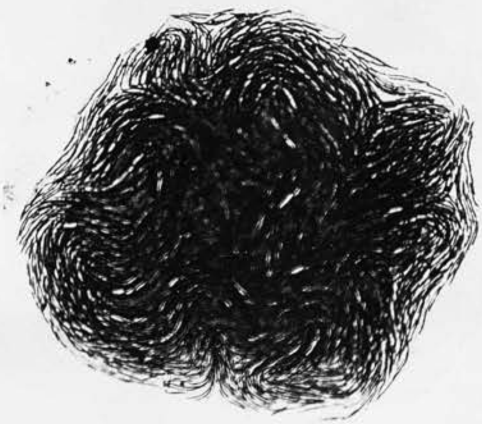
1. Smooth colonies. x 300.

2. Rough threads growing from smooth colony. x 650.

But on successive sub-culture, there appeared in cultures of one gravis and one mitis strain, stalked and/

and fan-shaped colonies, consisting of a dense nucleus from which long threads grew outwards on one side (Plates 2 and 3). On sub-culture, these strains produced radially symmetrical colonies, resembling those of the anthrax bacillus (Plate 4).





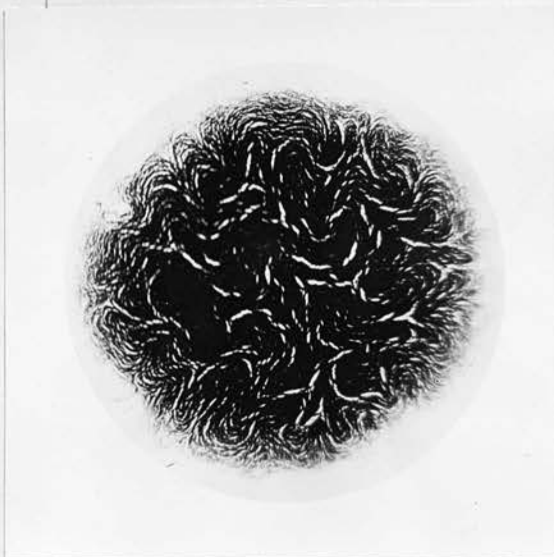
C. diphtheriae (mitis)

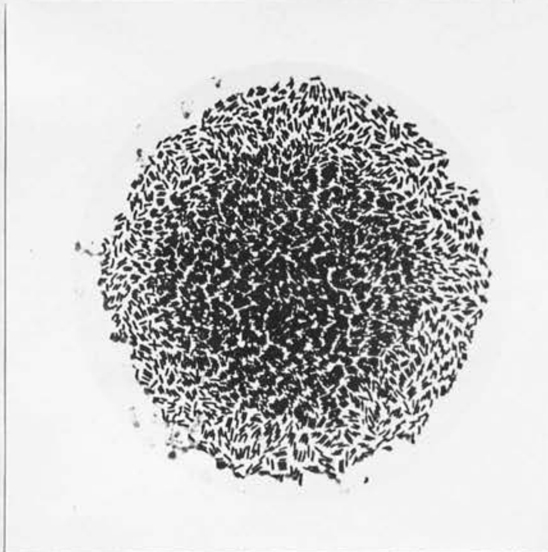
3. Rough threads growing from smooth colony. x 1000.

4. Rough colony. x 650.

A number of preparations were then made from colonies of S. dysenteriae and paradysenteriae and several strains of B. coli, from which it became evident that all colonies which were morphologically rough, possessed the structure of the "Medusa head" colony of the anthrax bacillus; whereas in those which were morphologically smooth, the individual bacilli lay/

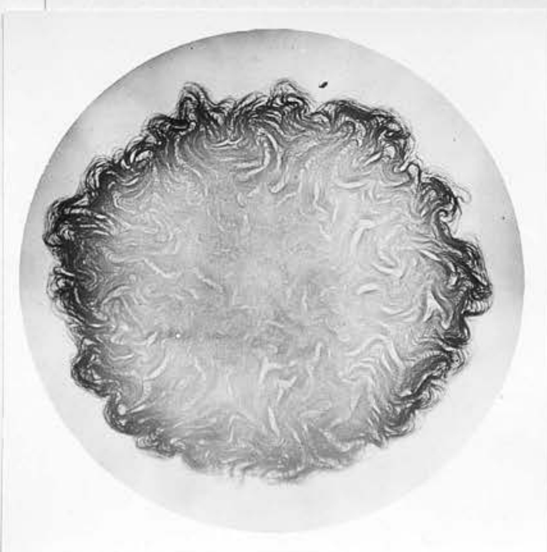
lay separately and without very much relation to one another (Plates 5, 6, 7 and 8).

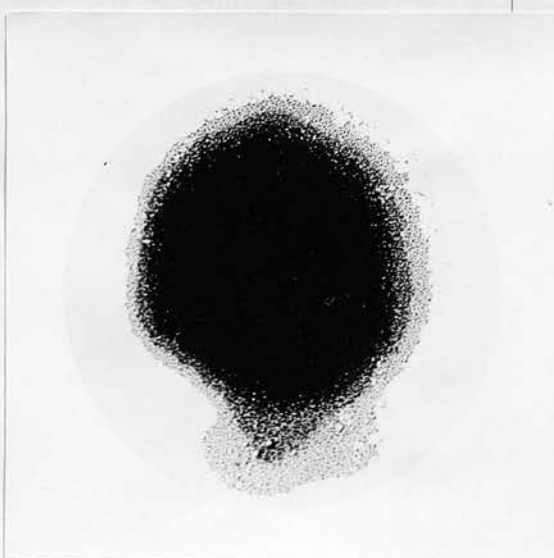




5. S. paradysenteriae (Sonne). Rough colony. x 100.

6. E. coli. Smooth colony. x 700.



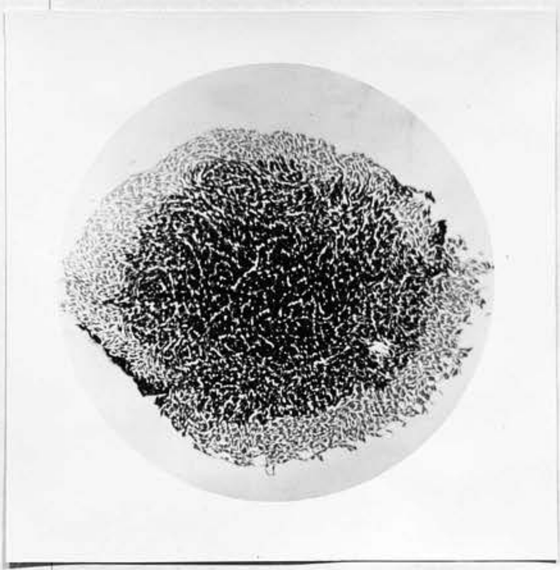


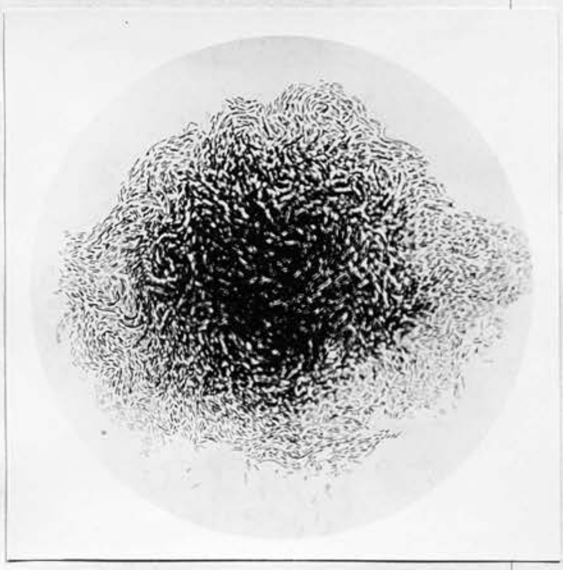
B. anthracis x 40.

7. Medusa head colony.

8. Vaccine Charbonneuse. Smooth colony.

The examination of an old laboratory strain of S. paradysenteriae (Sonne) presented a new problem. This strain invariably produced flat, smooth colonies on agar, and thin, spreading colonies, of a "ground-glass" appearance on MacConkey's medium. Although distinct in their appearance, these forms were essentially similar in their minute structure, consisting of parallel loops of a somewhat simpler appearance than those of an "anthracoid" rough, and composed of bacilli in chains rather than threads. The main differences between the two types appeared to lie in the fact that in the "ground-glass" rough colony the loops were loosely arranged, whereas in the first-mentioned colony they were closely knit, giving an appearance which tends to lead to confusion with genuine smooth forms (Plates 9, 10 and 11).





S. paradysenteriae (Sonne) x 200.
9. Pseudo-smooth colony. 10. Ground-glass rough colony.

Forms resembling this intermediate type of colony were subsequently discovered in cultures of E. coli, S. dysenteriae and later in B. anthracis, the majority of colonies in one strain of Vaccine charbonneuse being of this type (Plate 12).

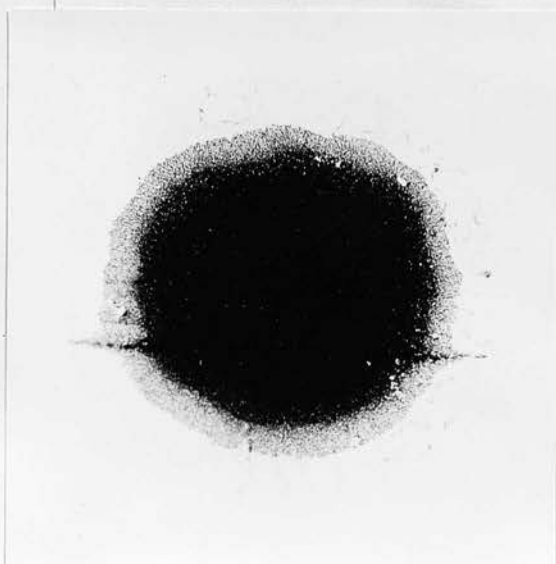
Preparations were made also from two virulent strains of B. anthracis and one strain of B. anthra:coides. These all gave colonies of the typical "Medusa head" appearance, which differed in no essen:tial detail from the morphologically rough colonies of the coliform organisms and C. diphtheriae.

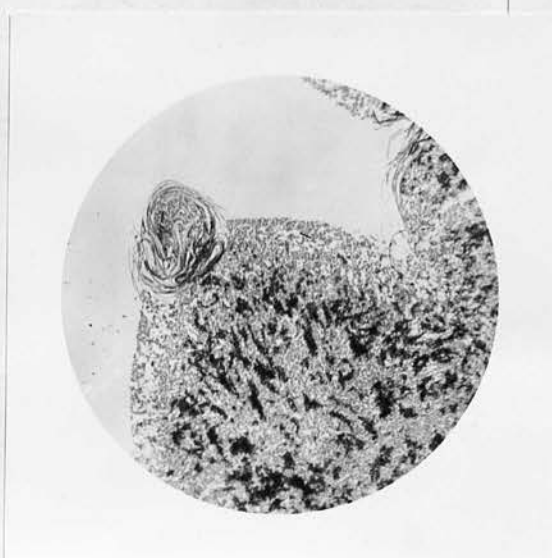
11. S. paradysenteriae (Sonne)
Portion of ground-glass
rough. x 650.

12. B. anthracis
intermediate
colony. x 150.

In order to induce variation, a strain of B. dysenteriae/

B. dysenteriae (Flexner) in the S phase (Plate 13) was grown in broth in the presence of an immune serum prepared against itself. When this was plated out upon agar, the culture grew in the form of large smooth colonies with satellite rough ones of a much smaller size, either attached or separate, and develop:ing sometimes in the form of a rough sector in a smooth colony, and sometimes as threads and loops growing out from the periphery (Plates 14, 15, 16).



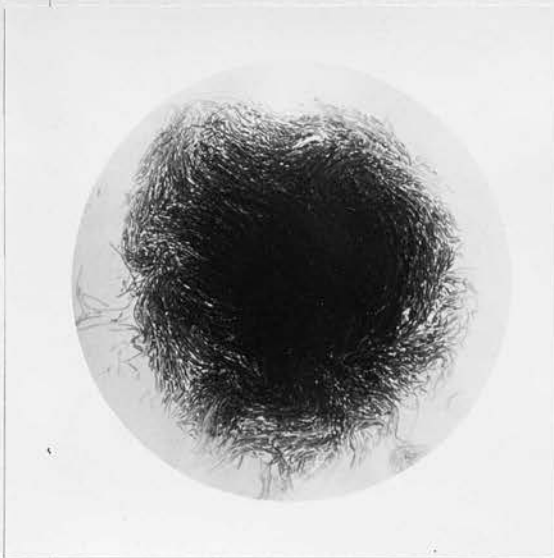


S. paradysenteriae x 100.

13. Smooth colony. 14. Rough sector in smooth colony.

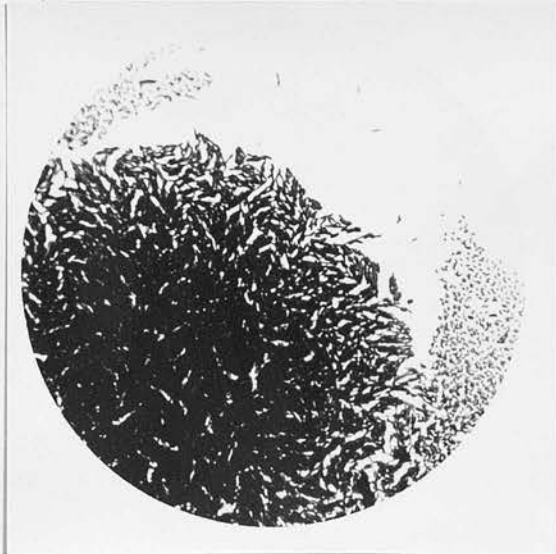
The differences appeared to be attributable mainly to the stage of development which had been attained by the smooth colony, when the rough character became evident/

vibrios of typical morphology (Plate 18).



Vibrio cholerae x 150.
17. Rough colony. 18. Smooth colony.

Between these extremes occurred an exceedingly interes-:ting form which produced colonies of moderate rough-:ness, composed of undivided spiral threads of regular length, about eight to ten times that of a single vibrio (Plates 19 and 20). These colonies were pro-:duced by a strain of El Tor vibrio of comparatively recent isolation, which had consequently retained its strongly curved or spiral morphology, whereas the older cultures had in many cases completely lost this appearance, being indistinguishable from coliform bacilli, both in the smooth and in the rough phase.

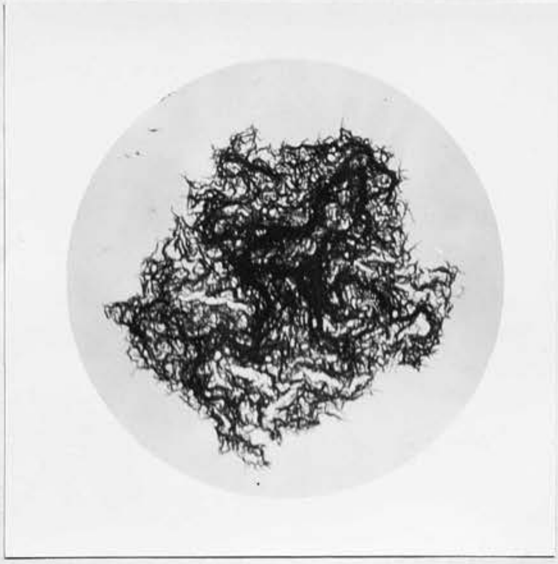



El Tor vibrio.

19. Intermediate colony x 300.

20. Intermediate colony, showing spiral threads. x 750.

Preparations were also made of colonies of <u>M. phlei</u> as a representative of the <u>Mycobacteria</u>. In these colonies, which were granular in appearance, the individual bacilli appeared to lie in a similar arrangement to those composing the smooth colonies of <u>C. diphtheriae</u>. At the same time, however, they showed a tendency to aggregate into clumps and ridges of growth (Plate 28). No variation from this form was observed.



21. M. phlei x 300.

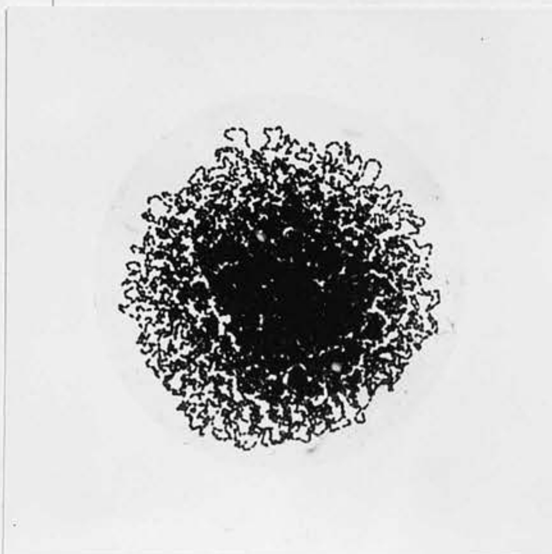
The Coccaceae.

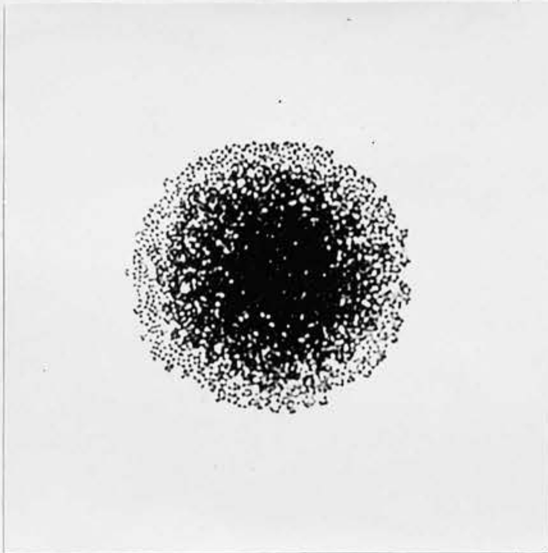
Colonies of various strains of streptococci and pneumococci were studied, the first to be examined being strains of Str. haemolyticus, some of which had been cultured for several years, while others were observed in primary culture.

Two main types of colony could be distinguished macroscopically; a disc-shaped form with a rough, granular appearance, and a smooth dome-shaped colony, sometimes "matt" and sometimes "glossy" in appearance. The rough form appeared, in most cases, to cause a more acute septicaemia in laboratory animals than the smooth; but/

but both forms occurred with approximately equal fre:quency on isolation from human cases.

The strains usually remained stable in their colonial forms, but dissociation from one form to the other was observed in several strains.





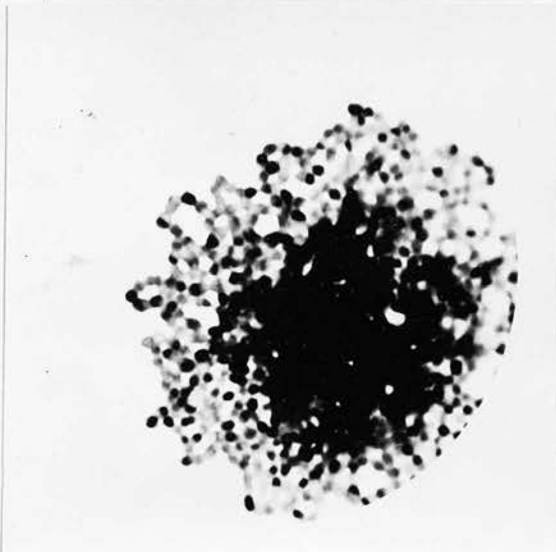
<u>Str. haemolyticus</u> x 500.

22. Long-chained colony. 23. Short-chained colony.

The microscopic structure of these forms was surprising in that the reverse of the conditions found among the rod-shaped species appeared to occur; that is the colonies of smooth appearance were composed of long chains forming loops at the edges (Plate 22), whereas in the rough forms the chains appeared to be much shorter, and looping was hardly observable (Plate 23).

The/

The appearance of the individual cocci was also different, those composing the rough colonies being much more lanceolate in appearance, and lying more widely separate from their neighbours in the chains than did those in the smooth colonies (Plates 24 and 25). In the case of the domed colonies there appeared to be no basic, structural difference between the "matt" and the "glossy" forms.



Str. haemolyticus x 1000.
24. Long-chained colony. 25. Short-chained colony.

In the pneumococci also, the two main types of colony were disc-shaped and dome-shaped, but owing to the polysaccharide capsule of this organism the structure was to some extent cloaked, and both types were smooth and mucoid in appearance. Apart from the morphology/

morphology of the pneumococcus the resemblance between these colonies and those of the streptococcus was very close (Plates 26 and 27).


<u>D. pneumoniae</u> x 650.
26. Long-chained colony. 27. Short-chained colony.

This resemblance was increased by the fact that those strains possessing the disc-shaped or "draughtsman" colony were found to be more virulent to mice than those with the domed colony and long-chained structure. Rough strains of pneumococci did not differ from these types except in the absence of the capsule; most of those examined belonging to the long-chained type.

When newly isolated, certain strains of pneumo-:coccus did not show a macroscopic appearance typical of either form, but the minute structure was almost always/

always distinctly either long-chained or short-chained, and with continued growth on culture medium the gross appearance became more recognisable as the disc- or dome-shaped form.

Although the long-chained streptococcus and pneumococcus colonies bore a considerable resemblance in their structure to those of a rough bacillary variant, they differed from it in that the peripheral loops were composed of single chains of cocci, while those of a bacillus were composed of numerous parallel threads. In a few strains of Str. viridans possess-:ing smooth, domed colonies, the chains lay in parallel strands, exactly like the rough bacillary form (Plates 33 and 34). Certain organisms of pneumococcal appear-:ance isolated from normal human conjunctivae were found to possess a domed colony the structure of which was intermediate between that of a long-chained pneumo-:coccus and a Str. viridans (Plate 28).

28. Coccus from conjunctiva x 300.

A few strains of staphylococci and of various species of Neisseria, some of which had been described as "rough" were also examined. No structure could be determined by the methods employed, except that the colonies of these strains differed from their smooth counterparts in being even more noticeably cohesive in appearance, so that in many cases no individual organisms could be discerned, the colony appearing almost as a homogeneous mass.

Other Criteria of Roughness.

When the above studies had been completed, the strains in question were examined for the property of auto-agglutination in saline solution. The majority of strains showing "anthracoid" structure were auto-agglutinable/

agglutinable in 0.85 per cent saline, but certain strains which were not otherwise distinguishable produced stable suspensions in this concentration, although agglutinating in a 3 per cent solution. In the case of the intermediate forms (vide supra), the pseudo-smooth form, as appearing upon ordinary agar, frequently gave an even suspension which flocculated slowly in large flakes, taking several hours to settle. Other strains with a similar colonial structure remained stable in a 0.85 per cent solution, but all of this type which were tested agglutinated rapidly in 3 per cent saline. Suspen:sions made from the "ground-glass" rough forms, as appearing on MacConkey's medium, were unstable in both solutions.

Agglutinating antisera were prepared both against a smooth strain of B. dysenteriae (Flexner), and against a morphologically rough strain derived from it, which did not agglutinate spontaneously in 0.85 per cent saline. With the homologous organism these sera gave agglutination to a titre of 1:960, and reacted to the same titre with the heterologous strain. Cross agglutination and absorption tests showed the rough and smooth variants to be antigenically identical. The morphological variation was not, in this case accompanied/

accompanied by the antigenic change with which it has generally been regarded as being associated.

Serum	Organism	Dilutions 1/30	1/60	1/120	1/240	1/480	1/960
S	S	++	++	++	+	+	±
	R	++	++	++	+	+	±
R	S	++	++	++	+	+	±
	R	++	++	++	+	+	±
S absorbed by R organism	S		-	-			
	R		-	-			
R absorbed by S organism	S		-	-			
	R		-	-			

DISCUSSION OF SECTION I.

Since the introduction of the terms S and R there has been confusion as to their exact connotation. The established importance of serological analyses has tended to make the evidence which these methods provide the determining factor in the ordination of bacterio-:logy. Consequently the antigenic change often associated with S → R transformation has come to be regarded as the most important part of the variation. Comparatively little attention has been paid to the problem of the structural alterations which are res-:ponsible for the observed differences in appearance.

Among/

Among the bacteria which have been studied, variation appears to occur in two distinct phases; a morphological change in the individual organisms, which usually affects, to a greater or less extent, the gross appearance of the colony; and a biochemical change, sometimes obviously affecting the surface or capsular material of the organism, sometimes observable only by chemical or serological methods, which usually affects the antigenic structure and virulence of the organism as well as the property of forming a stable suspension in saline solution, and which may, where the extent of change in the surface constitution is sufficiently great, alter the appearance of the colony. These two phases of variation may occur separately, but appear to be closely linked in their behaviour.

In the rod-shaped genera the fundamental, morpho-:logical change which takes place is that the individuals composing the colony cease to be short bacilli with rounded ends, having little attachment to their neigh-:bours, and become longer, with square-cut ends, by which they are strongly attached to one another. Thus the colony ceases to consist of a large number of separate organisms, and may become a single convoluted thread. Between these two extremes many intermediate forms occur.

It/

It is highly probable that the long-chained, domed, and the short-chained, disc-shaped colonial forms of the streptococci and pneumococci, with their respective close and loose attachment of the component individuals, are directly analagous to the morpholo-:gically rough and smooth bacillary colonies. The usually described S → R variation of the pneumococcus, and the "matt → glossy" variation of the streptococcus appear to be changes belonging to the second phase mentioned above, connected solely with the polysacc-:haride capsular material at the surface of the organism. It is this material which normally conceals the rough structure of the colony. The capsular material of the pneumococcus is more profuse and obvious than that of the streptococcus, but there appears to be little doubt that such polysaccharide surface material is possessed by the great majority of bacteria, and it is probable that in forms as closely related as the streptococci and pneumococci, similar variations are due to the same basic changes.

The existence of a parallelism between the strepto-:cocci and the bacillary forms, which does not appear to relate to the other groups of coccal species, is borne out by the observations of Wyckoff and Smithburn (1933), who stated that "true cocci" including the staphylococci/

staphylococci, enlarged in all dimensions before division, while the streptococci merely elongated. This point will be considered in a later section.

The conception that changes in colonial appear-:ance are closely associated with individual morphology, was enunciated by Baerthlein (1918) in his monograph on variation. Similar views have been expressed by Hadley (1927), while recently Smith (1931), working on the Haemophilus group, stated that variation from a cocco-bacillary to a filamentous form was always immediately associated with a change in colonial appearance.

In addition to those described there must be many other changes in morphology, capsulation, pigment production and other characters, which are responsible for the innumerable major and minor differences in colonial appearance which have been described, and which continue to be of great importance in the identi-:fication of many bacterial species. Within the scope of the present work there remains unexplained the cause of the apparent adhesiveness of the bacilli composing the colonies of M. phlei, and also the reason for the rugose appearance of certain colonies of the staphylococci and Neisseria. Taking into consideration/

consideration the exceedingly cohesive appearance of these latter colonies when examined microscopically, it is not improbable that this "roughness" may also be due to a change in the qualities of the polysaccharides at the surfaces of the organisms, as in the case of the other members of the <u>Coccaceae</u>. In this case however, these polysaccharides appear to behave as a cement-like matrix in which the cocci are embedded, or as an adhesive capsule by which the acid-fast bacilli are held firmly together in masses. It is certainly true that the strain of <u>M. phlei</u> from which the preparations were made yielded a high proportion of gummy polysacc-:harides on chemical fractionation (Author's observa-:tions), while as regards the staphylococci, differ-:ences in the constitution of these compounds as isolated from "smooth" and "rough" strains have been recorded by Hoffstadt, Youmans and Clark (1934) and Julianelle and Wieghard (1933-34).

SECTION II.

Modes of Growth.

Smooth Forms.

In the case of S colonies of the coli-typhoid organisms, it was seen that the very young colonies were not entirely devoid of structure, but that as they increased in age, this appearance became lost or masked, until the characteristic, apparently structure-:less appearance was attained.

This was apparently due to the fact that the longitudinal attachment of the bacilli was so slight that a chain of more than ten or twelve bacilli became instantly distorted by the growth of the constituent organisms, and hence the tendency to chaining was observable only in the very young colonies (Plate 29). f 44

The break-up of such chains as might have occurred was accompanied by the "slipping" movement mentioned by Graham-Smith, Nutt, Seal and others. But in this case, i.e. on the surface of the medium, the "slipping" was merely a matter of the growth of the organisms after division resulting in the two opposing ends being pushed past one another until portions of them lay over-lapping (Diag. 1). after f 58 A smooth colony in its early stages was seldom observed to be more than one or two organisms thick at the edges, with a "plateau" of greater/

FIGURE I.

GROWTH OF COLONIES OF VACCINE CHARBONNEUSE. I.

SMOOTH TYPE.

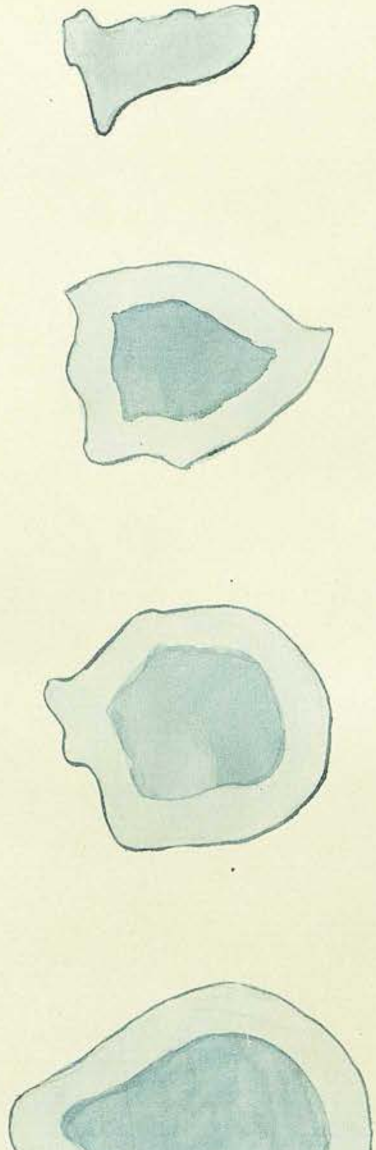
Observations at :- 3.30 pm.

4.30

5.30

9.30

Showing gradually decreasing irregularity of outline, and sharply bounded central thickening.



greater thickness commencing about a third of the way to the centre; the dividing line between the two portions being quite sharp and distinct (Plate 6 /23). With increasing age the thickness appeared to increase somewhat, but usually remained an infinitesimal fraction of the diameter, even in those smooth colonies which had the appearance of being markedly strongly domed. Such smooth colonies also showed irregularities of outline corresponding to minor irregularities in the surface of the medium, and in growth threw out exten-:sions in all directions, which gradually expanded and widened as the perimeter of the main part of the colony overtook them in its growth (Figure 1).

The units of which smooth colonies in their later stages, were composed, were usually bundles of from two to ten bacilli lying parallel, and showing little regularity of orientation to those composing other bundles. As far as could be observed, these bundles were not formed by any regular mechanism, but hap-hazard, as a result of the chance meeting of bacilli, and by "slipping" growth movements. Their subsequent adherence was, at least to some extent, the result of the natural tendency of rod-shaped bodies to keep their longer axes parallel when drawn together by surface tension. The occurrence of these bundles tended to give/

FIGURE 2.

GROWTH OF COLONIES OF VACCINE CHARBONNEUSE. II.

SMOOTH-INTERMEDIATE TYPE.

Observations at :- I2.30 pm.

I.30

2.30

3.30

4.30

give an irregular outline to some smooth colonies. But with an increase in the size of the colony, the relative size of the bundles decreased, and the irre-:gularity from this cause became less noticeable.

29. S. dysenteriae. Smooth colony. x 500.

30. S. paradysenteriae Primary coil. x 500.

Rough Forms.

Observations on the growth of rough colonies were more easily made, as in this phase, they appeared to follow a more definite line of development than in the case of smooth forms. During the initial stages of growth, occasional straight threads of considerable length were observed, but more frequently coiling or kinking of the threads occurred before much growth had taken/

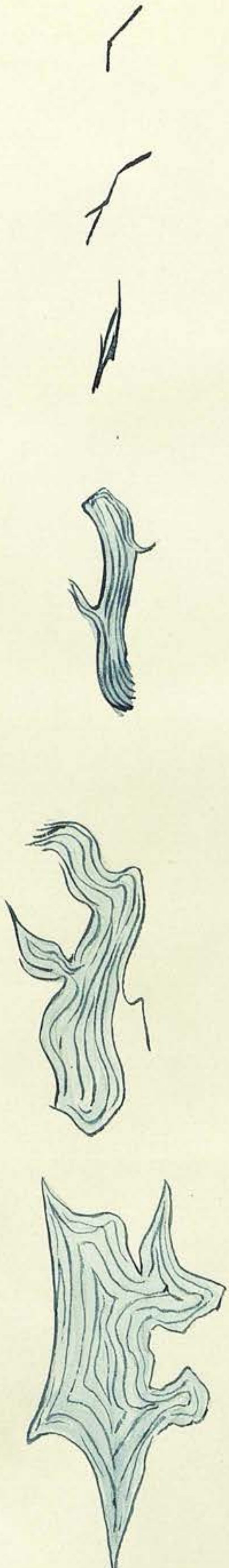
FIGURE 3.

GROWTH OF COLONIES OF VACCINE CHARBONNEUSE. III.

MIDDLE-INTERMEDIATE TYPE.

Observations at :- 2.30 pm.
3.20
4.0
6.0
7.30
9.30 am.

Showing formation of "primary group" before commencement of wreathing of the edge.

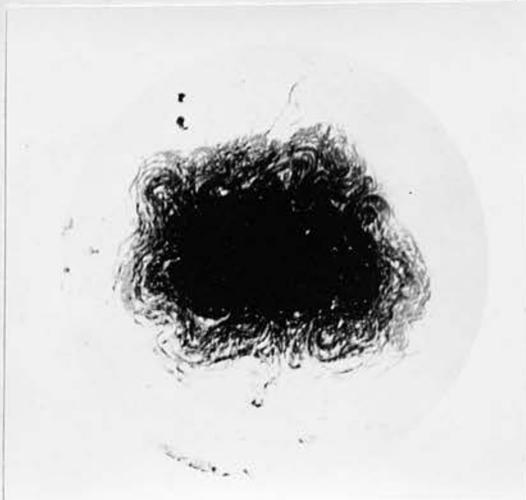


after f 58

taken place (Diag.II. 2 and 3). This was almost unquestionably due to the friction of the surface of the medium, against which the rigidity of the threads was no longer able to maintain their straightness in view of their constantly increasing length which led to the formation of a primary loop or coil, generally of a simple form (Plate 30; Diag.II. 4). During, and subsequent to the formation of this primary coil, all parts of the bacillary thread appeared to be in an active state of growth; this increasing elongation of all portions of the primary coil was accomodated by the formation of a number of folds (Plate 31), fre-:quently comprising all the "turns" of the bacillary thread composing the coil, giving the colony the appearance of a Maltese cross (although the number of arms was usually greater than four), (Plate 32; Diag. II. 5).

* this is the f 44? after f 58

4 f 58



S. paradysenteriae.
31 and 32. Infolding of primary coil. x 300.

FIGURE 4.

GROWTH OF COLONIES OF VACCINE CHARBONNEUSE. IV.

ROUGH-INTERMEDIATE TYPE.

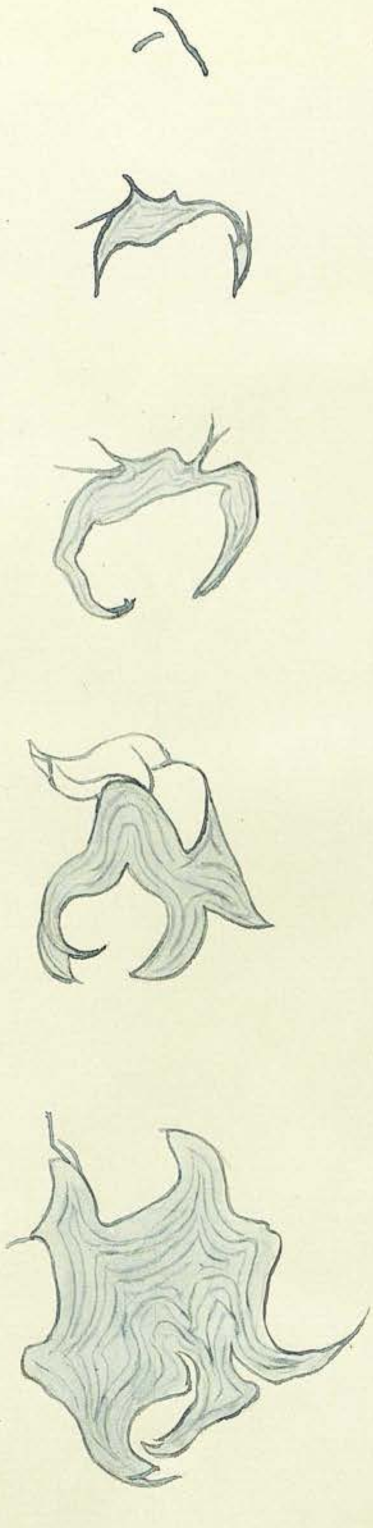
Observations at :- 2.30 pm.

4.I5

5.I0

6.I5

7.5



Beyond this stage it could not be observed whether the interior portions of the colony continued to grow with the same rapidity as did the exterior portions, but continued growth of the latter led to the formation of secondary folds both on the out- and inturned portions of the original folds, thus rapidly destroying the simple appearance of the colony (Diag.II. 6 and 7; Figures 5 and 6).

Variations of this method of growth occurred almost entirely in the early stages, particularly in the mode of formation of the primary coil, which was also profoundly influenced by the number of individual bacilli which took part in it (Figures 7 and 8).

It should be emphasised that only in the minority of cases were colonies observed to grow from single bacilli, such isolated organisms frequently failing completely to grow, or growing only after a prolonged lag phase; whereas others in more heavily inoculated areas of the plate, grew more readily, even though they were separated by as much as 20-50μ from the neighbouring organisms.

Rough colonies seldom reached any size without numerous off-shoots consisting, at first, of a single bacillary thread or a very small number of such threads growing outwards from the edge of the colony. Such off-shoots/

FIGURE 5.

GROWTH OF COLONIES OF VACCINE CHARBONNEUSE. V.

ROUGH TYPE.

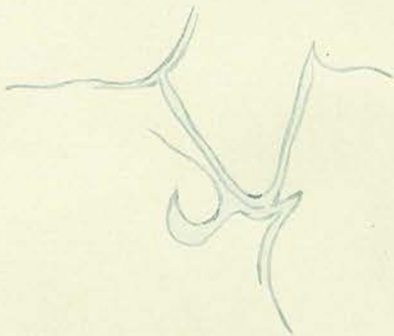
Observations at :- 2.15 pm.

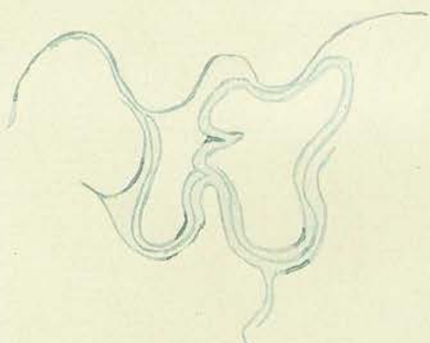
3.40

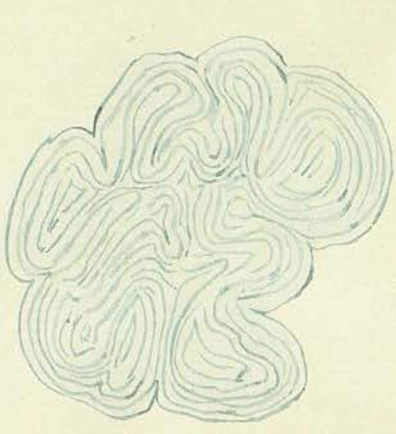
4.45

6.0

Showing formation of "primary coil" from a group of bacilli.







off-shoots, however, rapidly underwent the fate of the primary threads and eventually became mere lobes of the colony as it grew and advanced outwards (Diag.III). aft p58 [illegible]

Except towards the centre of the colony the con-:stituent threads of the coils did not appear to over-:lie one another to any extent, and usually adhered firmly to the surface of the plate, under the influence of intermolecular attraction, so that the ultimate thickness of the colony was always, as in the case of smooth types, an infinitesimal fraction of its diameter (Diag.IV). [illegible] aft p58

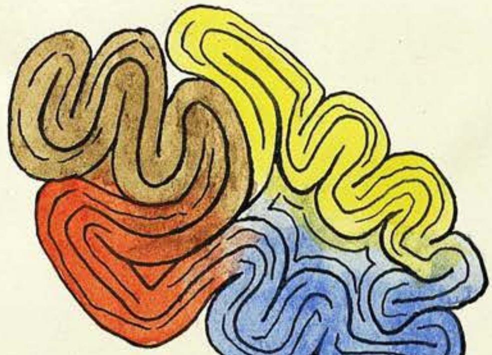
The formation of the widely looped colonies of the streptococci differed in one respect from those of a rough bacillary form in that the units composing the chains were so small that the chains themselves were much more flexible than the bacillary threads; thus frequently the loops were composed of a single chain, and were consequently very numerous around the periphery of the colony.

FIGURE 6.

GROWTH OF COLONIES OF VACCINE CHARBONNEUSE. VI.

ROUGH TYPE.

The same colony in two stages of growth. (16 hrs.) Showing the increasing convolution of the individual lobes produced by elongation of the component threads.



Str. viridans
33 and 34. Development of colony. x 300.

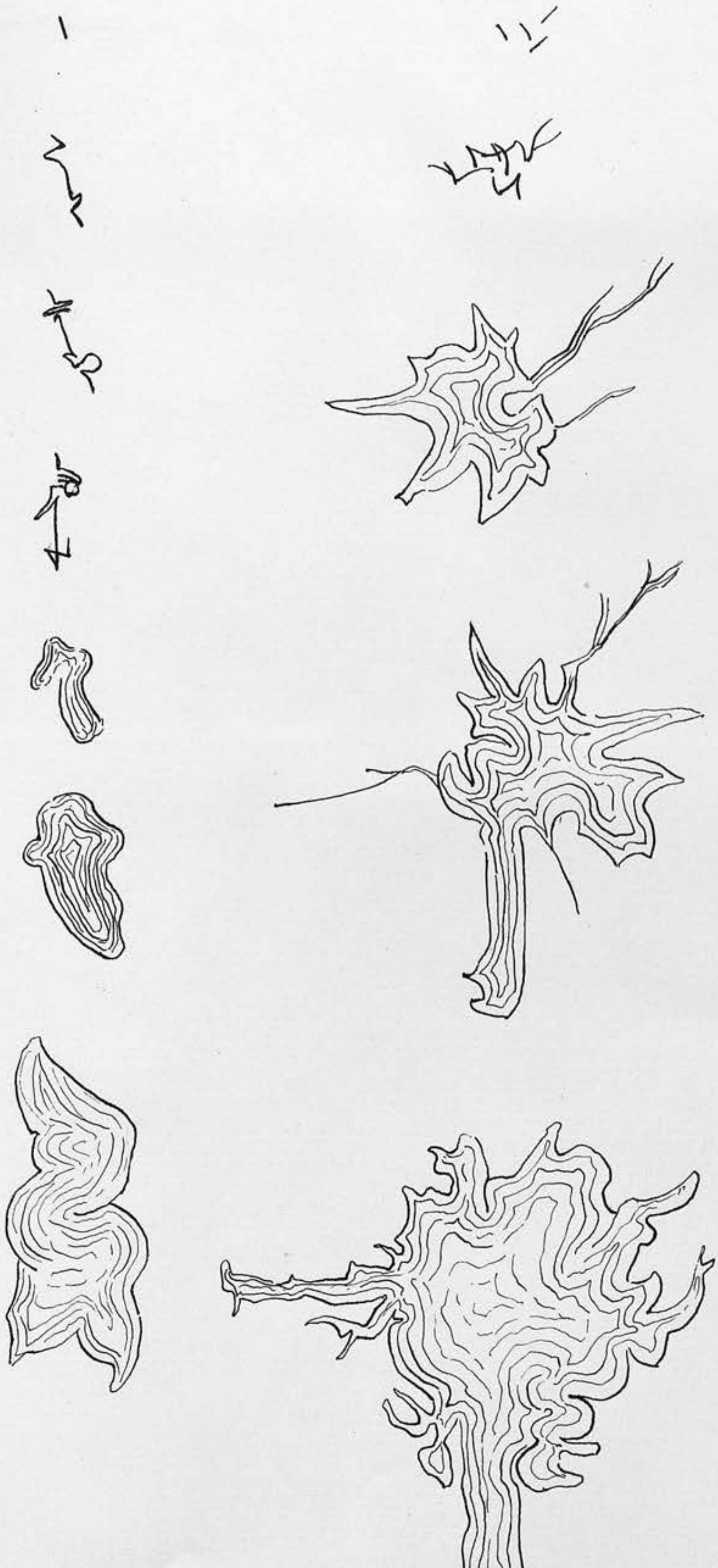
This was not invariably the case, and some strains - particularly those of Str. viridans were almost indis-:tinguishable microscopically from typical "Medusa Head" colonies (Plates 33 and 34). Such cocco-bacillary forms as Past. pseudotuberculosis which were examined, were intermediate in this respect, and pro-:duced coils of a varying number of chains (Plates 35 and 36).

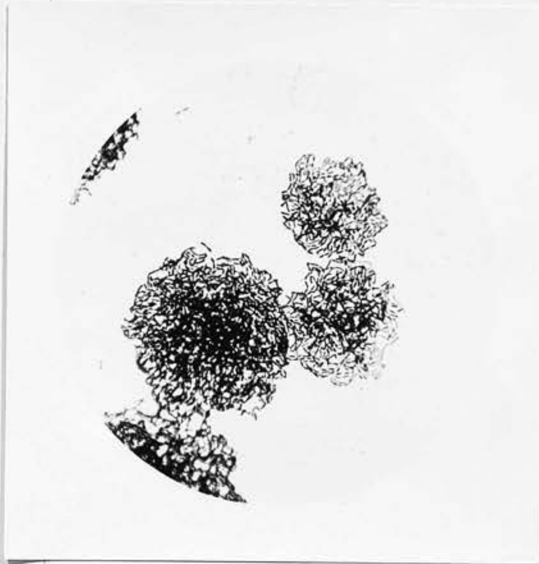
GROWTH OF COLONIES OF <u>BACILLUS ANTHRACOIDES</u>.

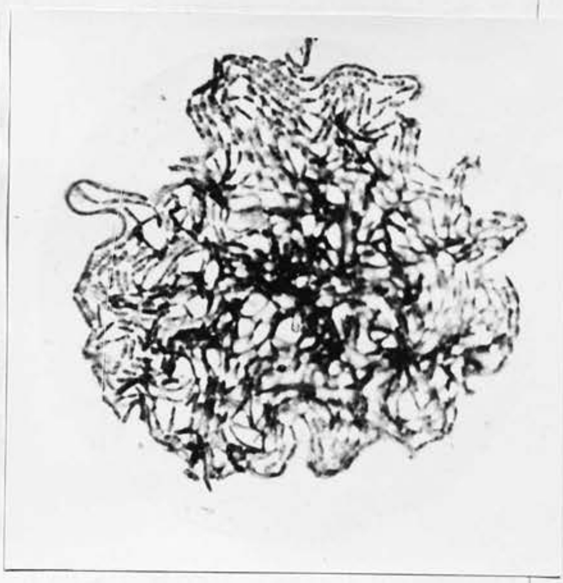
FIGURE 7.	FIGURE 8.
From a single bacillus.	From a group of bacilli.

Observations at :-

II pm.	2.40 pm.
II.50	3.0
I2.I5	4.20
I2.30	5.40
I2.45	8.I5
2.20	
3.I5	







P. pseudotuberculosis

35. Colonies. x 300. 36. Colonies. x 1000.

Intermediate Types.

The strains of coliform organisms and of Vaccine charbonneuse which were used showed all stages of variation between the truly rough and smooth forms. In the majority of these "intermediate" types there was a certain amount of "slipping" and irregular growth until a primary group of a certain size was formed, beyond which stage the colony increased in size by folding and wreathing to a greater or less extent according to the "roughness" of the strain (Figures 2, 3 and 4). The size which the colony was forced to attain before any noticeable folding of the edge/

edge could be indulged in, also depended upon this factor, and even beyond this point any large irregu-:larities which formed were usually broadened and rounded out by the pressure of growth from the centre, as in the case of smooth colonies, and any indentation filled in from within; the strength of the threads or chains seldom appearing to be strong enough to allow of much divergence from the simple outline of the colony, even on the part of quite substantial projec-:tions.

B. mycoides.

A number of observations were made of the mode of growth of B. mycoides which appeared to be so much more rigid than even an ordinary rough bacillary variant that the threads were enabled to thrust their way outwards to considerable lengths without kinking or breaking. The end of the thread, whose component bacilli were all simultaneously elongating could be observed to move with the accumulated velocity of all these units, traversing the field of the microscope with great rapidity. Even in the case of this organism, however, there was a limit to which the threads could extend, and in well-grown cultures, coils and loops, resembling the primary coils of rough colonies were found at the ends (Plate 37).

37. B. mycoides formation of coils x 50.

Mode of Division.

An attempt was then made to observe the mode of division of the individual organisms composing these colonies. These observations were made with the oil-immersion lens, on bacteria growing under cover-slips on the surface of semi-solid agar in watch-glasses. The first organism to be studied in this manner was B. mycoides, which was chosen because of its large size, and because it possessed, in an exaggerated form, the characteristics of a rough bacillary variant.

In this species the first sign of division about to take place was the sudden appearance of an extremely fine/

fine line across the organism. This appearance was followed in a few minutes by the apparent formation of a slight concavity at one side of the dividing line, and a slight convexity at the other, giving the appearance of a ball-and-socket joint; after which the edges of the "socket" retracted until this side of the division also was slightly convex, thus completing the division - the whole operation taking from five to fifteen minutes (Diag. V).

In the case of the anthracoid bacilli which were then studied, this sequence of events was frequently much less clear, and was preceded in some cases by a swelling at the point of division, possibly indicative of a weakening of the envelope at that place; and in others by a certain amount of constriction, forming a very narrow, if occasionally quite a deep groove around the bacillus. The behaviour of the smooth and rough strains in this respect was distinguishable mainly by the greater tendency to constriction shown by the smooth strains, the intermediate types being intermediate in this as in most other respects. It was, however, most noticeable that the division of the smooth forms was into two equal portions, while it was rare for the rough strains to divide equally, the formation of a large and a small bacillus being more common/

common; the smaller one frequently growing so rapidly as to overtake the greater, and be the first to subdivide (Diag. VI). The rough forms appeared to be capable of dividing at any point, and division fre-:quently occurred at some point which was subjected to external interference; usually by pressure from another growing bacillus (Diag. VII).

Here also, no internal structure could be observed in the dividing bacilli, but in the smooth forms whose division was more regular in position, there were fre-:quently observable a large number of granules, which congregated mainly at the ends of the bacilli, and when these bacilli were about to divide, migrated to some extent, to the point of division.

It is interesting to note than when a smooth culture of Vaccine charbonneuse exhibiting such granu-:larity had become aged, the granules began to disappear, simultaneously the organisms ceased to divide, and began to grow in the form of undivided threads of very slight rigidity, which accordingly became highly con-:voluted as they grew in the confined space under the cover-slip (Diag. VIII). It was not uncommon to find a bacillus, one half of which was rigid and granular, and the other a non-granular and convoluted filament.

Similar/

Similar observations were made upon strains of <u>Str. haemolyticus</u> of both the major colonial types, i.e. long-chained and short-chained, and upon strains of <u>Staph. aureus</u>. All these Coccaceae divided by elongation in one dimension, and division of the resultant oval organism into two cocci of the appear-:ance of Neisseria organisms (Fig.); no significant difference could be observed between the modes of division of the different streptococcal types, and none of the cocci showed any trace of septum formation opp. f.58 (Diag. IX).

"Snapping" and "Slipping" Movements.

The technique used for the above observations was additionally interesting in that it was essentially similar to that of Nutt and Seal. In this case also, even in the comparatively soft agar used, as soon as there had been sufficient growth to crowd the bacteria together, the "slipping" and "snapping" appearances were observable in the smooth and rough strains opp. f.58 respectively (Diag. I and X).

DISCUSSION OF SECTION II.

The relationship between the morphological changes and the alterations in colonial structure which they produce, appears to be of the simplest possible/

possible character, depending mainly on the previously-mentioned differences in firmness of the attachment of the component organisms to their neighbours in the colony. This varies from practically <u>nil</u> in the case of the completely smooth forms, to the very great rigidity of <u>B. mycoides</u>. In addition to these types there are those such as the long-chained form of <u>Str. haemolyticus</u>, in which the units of the chain are strongly but flexibly attached. These qualities and differences are manifested in the form of colonial structure, under the influence of the external forces supplied by the surrounding material.

In the first place the bacteria are held closely to the surface of the medium by intermolecular attraction, (the so-called solid medium being, in fact, a colloidal gel), and secondly, the growth, and consequent extension of the bacterial threads and chains across the surface of the medium is impeded by friction, which causes them to increase in length by the formation of loops and coils, rather than by direct extension in a straight line. Where the longitudinal attachment is very slight, as in the case of smooth bacillary variants, and short-chained streptococci, these loops and coils either do not occur, or do so only to a limited/

limited extent - the bacteria being, for the most part, pushed past one another in the "slipping" motion as the chains elongate.

The observations of Nutt and Seal on the modes of growth of R and S variants, which were made upon organisms growing in agar under coverslips, were perfectly correct as far as they went. The applica- :tion, however, of these observations made upon bacteria growing under conditions of severe mechanical restraint, to the case of the same organisms growing in comparative freedom on the surface of the medium, is not valid. And as far as these results are applied to surface colonies they are misleading. Graham-Smith, in his classical studies on bacterial growth, appears to have realised this, but did not take sufficient precautions to ensure the avoidance of this restraint.

In the case of a rough strain growing under the first-mentioned conditions, the bacillus would attempt to grow outwards and elongate. Meeting with immediate resistance from the medium in which it was embedded, it would fracture at the point of division and produce the snapping growth described by these workers. In the same manner, the two daughter cells resulting from the division/

division of a smooth variant would be forced back past one another to lie side by side, as soon as their growth approached the size of the original bacillus. Surface growth, however, is not restrained to the same extent, and the development of the colonies is as described above.

The studies on the actual division of the organisms presented a new problem. There appears to be a grada-:tion in the complexity of the process, ranging from the elaborate septum formation of B. mycoides to the simple constriction and fission of the smooth forms. On the other hand the occurrence of division in B. mycoides and the rough variants appears to be much more irregular and uncontrolled, while in the smooth variants it is regular, and even shows signs of being controlled by a definite mechanism, which may corres-:pond to the diffuse nucleus described by Guilliermond and others. In view of the apparent fact that com-:plete division is the normal mode of life among the smooth, but not among the rough variants, this second state of affairs is not surprising, and it may be that the division of the rough forms is more closely allied to the irregular septum formation of the mycelial threads of certain types of fungus.

It/

It is interesting to observe that just as the behaviour of the streptococci resembles that of the rod-shaped genera in the matter of colonial form, so is the mode of division essentially similar, being simply that of a very short bacillus, as reported by Wyckoff and Smithburn, while the form adopted by such cocco-bacillary species as the Pasteurella group provides a verification of this similarity between coccal and bacillary forms, being midway between that of a streptococcus and a rough bacillus, just as the individual organism is intermediate between these types.

Although the conclusions drawn from these obser:vations may tend somewhat towards the view that bacteria are a homogeneous group in many respects, they indicate that similarity of colonial structure, such as the resemblance between the rough colony of a dysentery bacillus and a Str. viridans, does not necessarily indicate close relationship, as this structure depends upon two factors, of which only one is intrinsic to the organism, and it is a simple matter for these factors to be fortuitously identical for species of widely different nature.

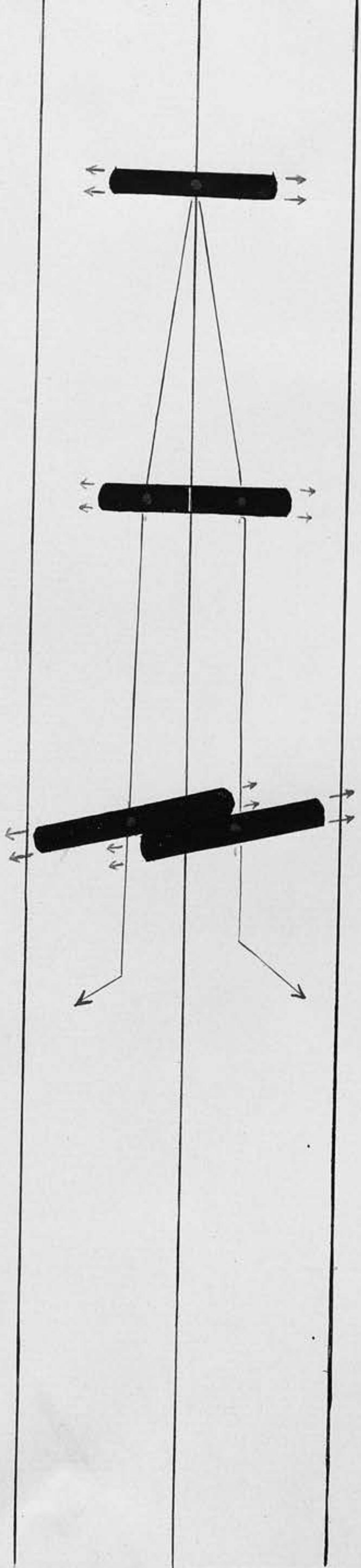


DIAGRAM OF THE GROWTH OF A

ROUGH COLONY.

I. Original bacillus.	Framework of diagram.
2.& 3. Extension and kinking of	"
bacillary thread.	"
4. Primary coil.	"
5.6. &7. Primary and secondary	"
infoldings of coil.	

1.

2.

3.

4.

5. (Reduced Scale)

6. (" ")

7. (" ")

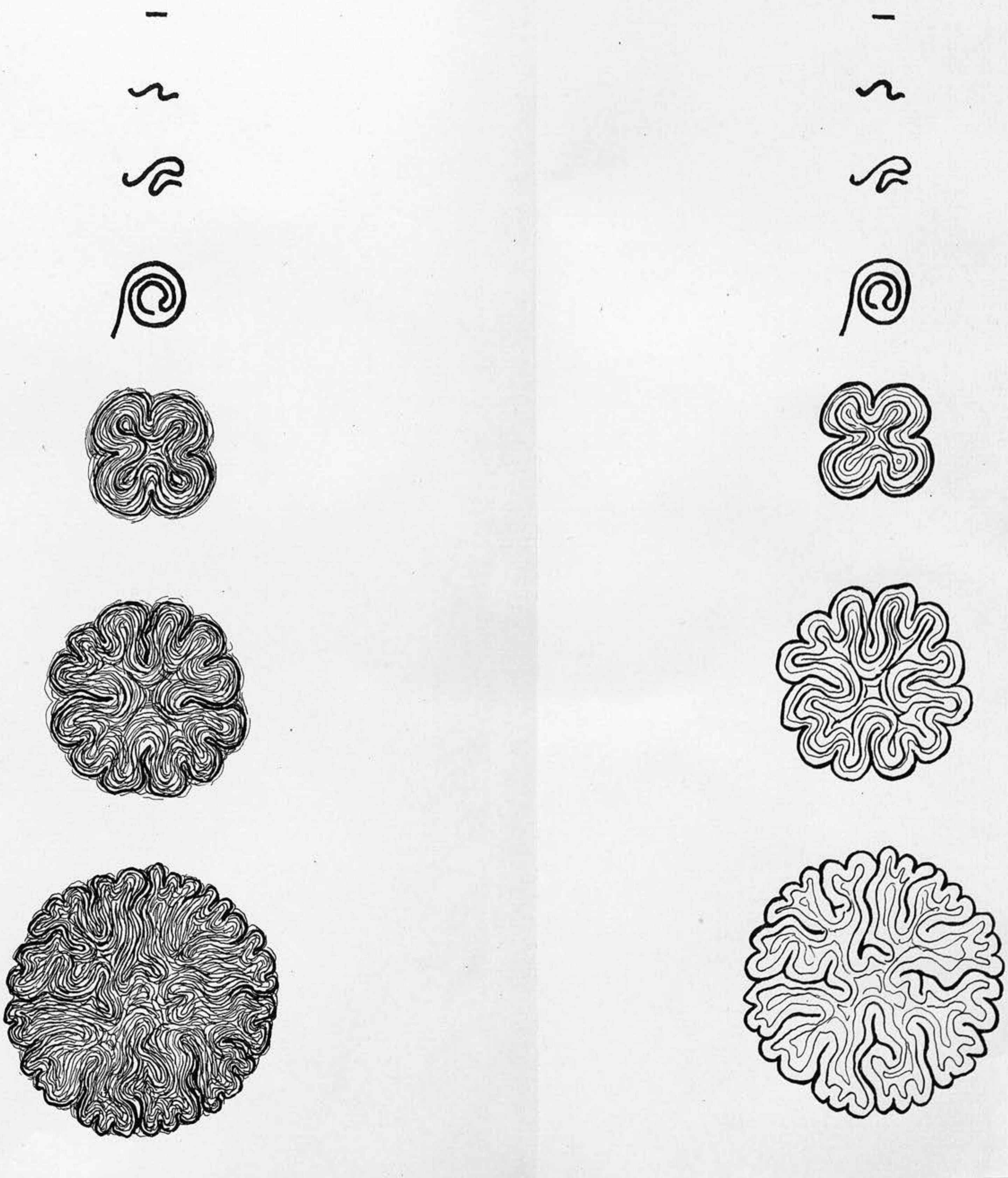


DIAGRAM OF THE GROWTH OF

A ROUGH COLONY.

Showing the transformation of an off-shoot into a lobe of the colony.

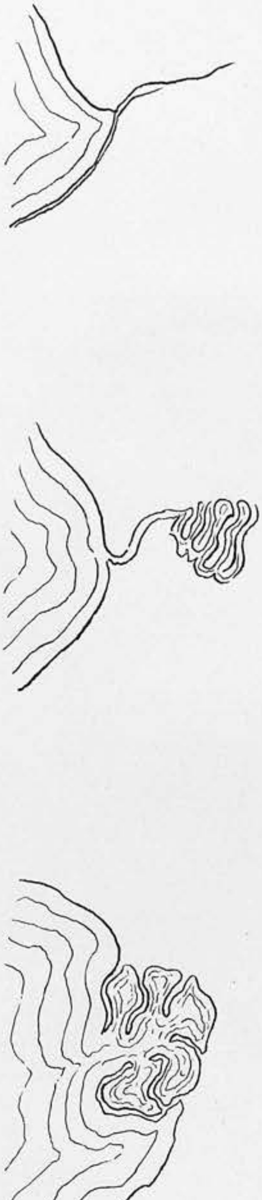


DIAGRAM IV.

DIAGRAM SHOWING THE REASON FOR THICKENING TOWARDS THE CENTRE OF A ROUGH COLONY.

Above, Plan; Below, Vertical Section.

Overlying of threads towards centre of colony.

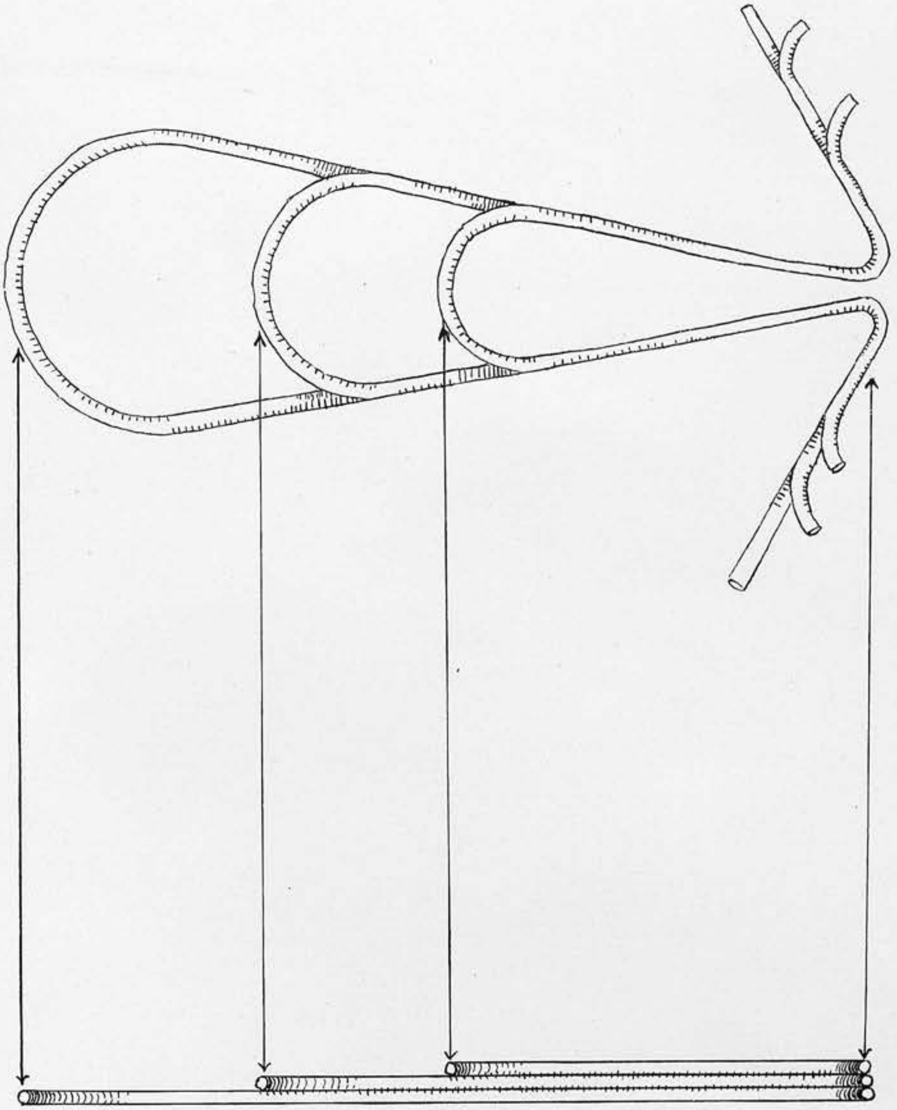
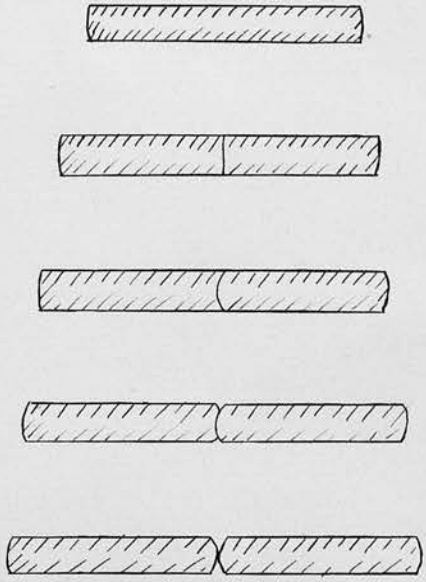


DIAGRAM V.

DIVISION OF BACILLUS MYCOIDES.

DIAGRAM VI.

DIVISION OF "ANTHRACOID" BACILLUS.



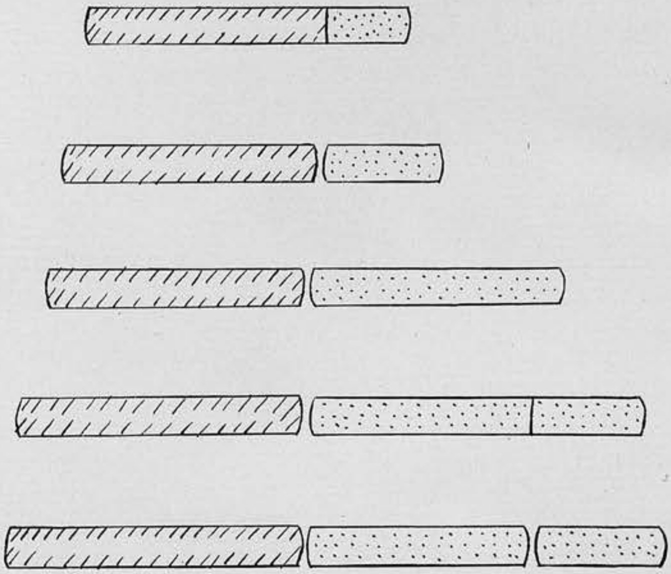
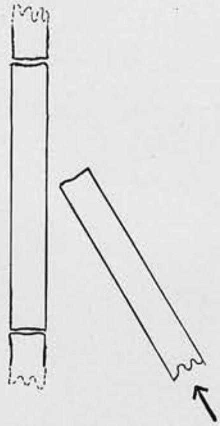
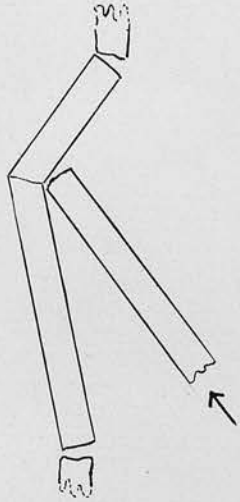


DIAGRAM VII.

DIVISION OF "ANTHRACOID" BACILLUS.

Showing the effect of external interference.





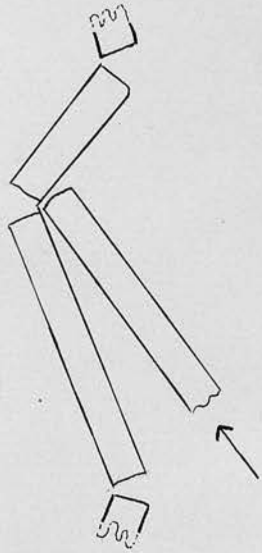


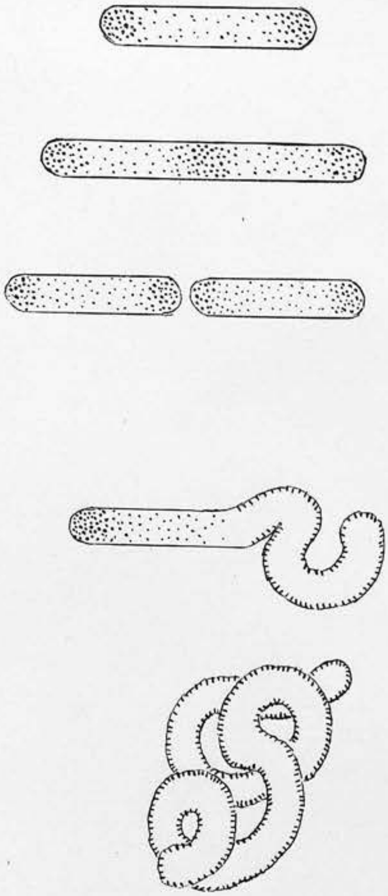
DIAGRAM VIII.

VACCINE CHARBONNEUSE.

Division and variation of granular, smooth type.

DIAGRAM IX.

DIVISION OF STREPTOCOCCUS HAEMOLYTICUS.



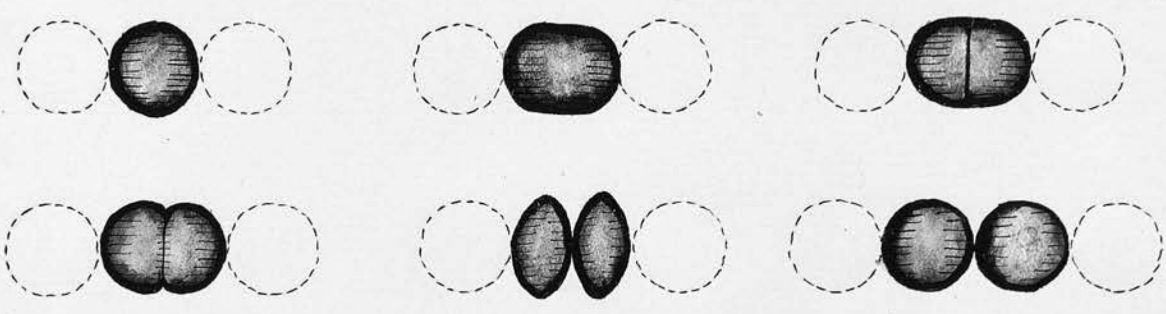
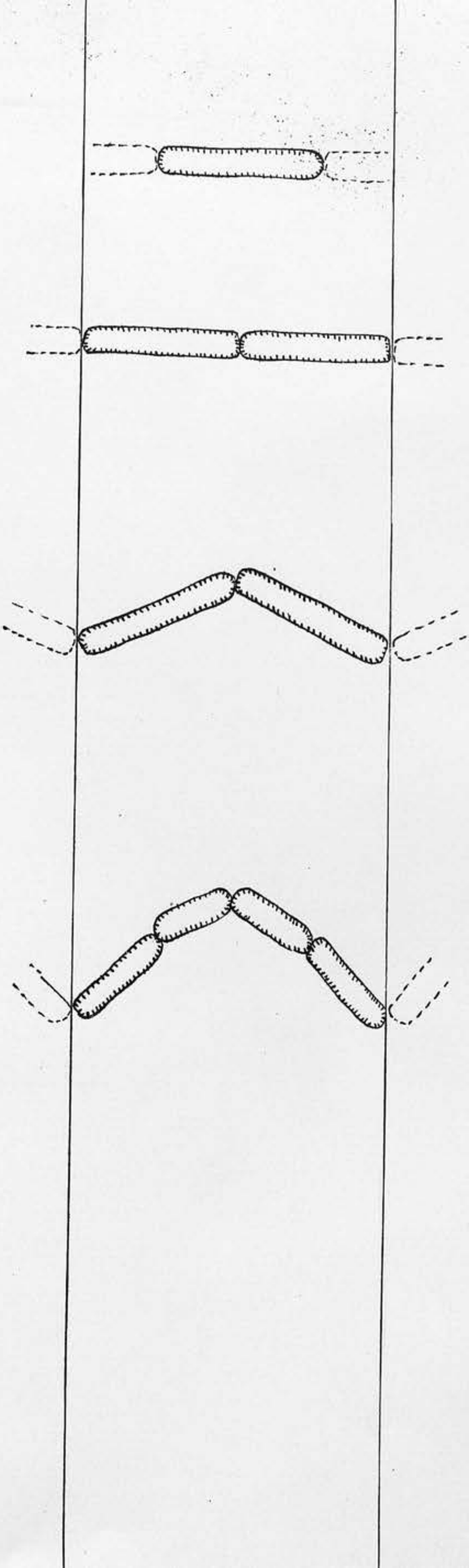


DIAGRAM X.

DIAGRAM OF "SNAPPING" MOVEMENT OF

ROUGH BACILLUS.



GENERAL DISCUSSION.

The studies in this thesis have been mainly concerned with the minute structure of the bacterial colonies described as "smooth" and "rough", which are generally regarded as true biological variants within a bacterial species. In the great majority of pathogenic bacteria, the smooth type is found present in the active disease, whereas on continued artificial culture, i.e. when the organism has become fully adapted to a saprophytic existence, transformation to a rough type is of frequent occurrence. It is of further significance that this latter type tends either to be avirulent or substantially less virulent than the former. In the case of the anthrax bacillus however, this state of affairs is reversed, the rough being the virulent, and the smooth the less virulent form. In the streptococci, as indicated above, there seems to be little connection between virulence and colony structure.

The writer's observations have served to emphasise a further general rule in relation to these changes, namely that among the bacillary and vibrionic species the rough colony is composed of convoluted filaments, either continuous or divided, while the units composing the smooth colonies are short, with rounded or even pointed/

pointed ends, and completely separated from one another. It may be added that while in certain species the smooth form is motile, the rough has lost this property. Among the streptococci the distinction between the individual cocci of the two colonial forms corresponding to rough and smooth is less marked, but distinctly analagous differences may be observed.

Taking all these features into consideration, it might be said that in some respects the rough is more "fungal" in morphology than the smooth. This is well illustrated in the work of Barber (1939) on variants of Erysipelothrix and Listerella. Thus, the filamen-:tous form of the former species has been taken to indicate a close relationship to the Streptothrices, but it is apparent from this recent work that it actually represents a "rough" variation. This leads to the question whether the filamentous variants of the bacillary species do in fact indicate a close relationship with the higher bacteria and fungi proper. In this connection reference may be made to one of the early views of the biological relationships of the bacteria, that they represent oidial forms of true fungi (see Muir and Ritchie, 1937). It is generally accepted that the yeasts are in fact fungi which exist permanently in this oidial condition (Fuchs, 1926; Henrici/

Henrici, 1930), and it has also been suggested by Lieske and Vuillemin (as quoted by Henrici) that the Eubacteria bear an analagous relationship to the Actinomyces. In addition to this oidial fragmenta:tion of the mycelium of these organisms several workers have observed the dissociation from certain strains (usually aerobic and non-pathogenic) of stable variants resembling diphtheroid bacilli (Nepomnaschy, 1930; Truiss and Politowa, 1931; Wright, 1937; and others). The bacillary types usually form soft, mucoid colonies, quite unlike the tough tenacious growths produced by the typical mycelial strains. If this variation is identical with that which dissociates filamentous and bacillary forms in the Eubacteria, it may well be taken to signify a complete absence of demarcation between the two main divisions of the bacteria, and at the same time emphasises the essential morphological difference between the smooth and rough phases.

From the immunological standpoint, much attention has been paid to the antigenic changes frequently associated with the S $\rightarrow$ R transformation, and this undoubtedly indicates a profound alteration in the chemical constitution of the bacterial cell. This alteration/

alteration appears to be closely associated with that which underlies the change in virulence. It is not proposed to discuss this aspect, except in so far as the virulence of the organism affects its mode of life, but rather to consider the possible biological significance of the morphological changes accompanying the so-called S $\rightarrow$ R variation of bacteria, which have been the subject of the studies recorded in this thesis.

As the relationship of the phenomena of variation to the problem of the general biology of these organ-:isms has been the subject of much study, a brief review of this question may not be inappropriate in an attempt to illustrate the findings recorded in the thesis.

In the majority of classes of living creatures, either within the life of the individual, or in other cases in the course of a few generations, the morpho-:logy and mode of life may pass through such a variety of modifications that individuals in different phases of their life-history may be entirely unrecognisable as members of the same species. Such life-cycles, when carefully investigated, have been of the utmost importance in the ordination of views on the evolution of many different genera, and in the elucidation of unsuspected relationships; while in some cases they have/

have been the means of proving that certain forms of life, long regarded as primitive, are in fact closely related to, and in all probability descended from more complex creatures.

The first detailed reports of the existence of similar phenomena in the bacteria were the studies of Almquist (1893, 1904, 1911) who claimed that the previously-held views on the simplicity of bacteria were erroneous; basing his arguments both on studies of pathogenic organisms and on his disbelief in the ability of what he called the "contact theory" to explain all the problems of epidemiology. Almquist believed that the life-cycles of pathogenic bacteria contained saprophytic as well as parasitic phases, and that multiplication took place by the production of minute, filterable gonidia, as well as by simple fission. His work however has not been widely accepted, and the first bacterial species to be generally recognised as possessing a true life-cycle, was the organism of bovine pleuropneumonia (*Asterococcus mycoides*), which was described by Bordet (1910), Borrel *et al.* (1910) and by other workers subsequently (Ledingham, 1933; Kleineberger, 1934; Turner, 1935), while later Jones (1913, 1914, 1920), Löhnis and Smith (1916, 1923) and Löhnis (1926) described an exceedingly complex/

complex cycle in Azotobacter. At the same time Löhnis made similar claims for a large number of other species.

It is interesting to observe that while the life-cycles of Asterococcus mycoides and Azotobacter were accepted almost without question by bacteriologists, the similar claims made by these and other workers (Maher, 1904; Bergstrand, 1920, 1921; Mellon, 1920, 1921, 1925; Haag, 1927; Hadley, 1927, 1928, 1937; Sweany, 1928; Ramsine and Milochevitch, 1928; Stoughton, 1929, 1932; Kahn, 1930; Schmidt-Kehl, 1930; Cunningham, 1930-1; Wyckoff and Smithburn, 1933; Wyckoff, 1934) for forms belonging to almost all the known genera and groups of bacteria have been regarded with dubiety.

These cycles as described by the workers noted above and by many others, to which may be added that of the organism of contagious agalactia (Bridre and Donatien, 1923; Wrobelewski, 1931) usually resembled one another in the occurrence of a series of changes in the morphology of the organism in question, the forms ranging in some species from long filaments to micrococci. In addition to these changes two other important phases of growth have been described in a great many species. These are the "symplasm" or "zooglea" in which numerous individuals congregate into/

into a shapeless mass of protoplasm from which new bacteria arise; and the previously mentioned gonidial or granular phase, which consists in the fragmentation of bacteria or portions of them, into tiny bodies similar to the spores of fungi, which germinate and reproduce the parent forms, sometimes after a period of simple multiplication resulting in an increased number of granules. Both these forms appear to be filterable upon occasion, and capable of regenerating typical bacteria after filtration.

In addition to direct morphological studies on the subject, these findings may be borne out, to some extent, by two phenomena which have been in themselves the cause of much controversy. The first of these is the production of filterable forms of the tubercle bacillus. Tuberculous lesions in which no bacilli could be demonstrated were first described by Mallassez and Vignal (1883), under the title of "Forme ou espèce de tuberculose sans bacilles", in which granular or micrococcal forms were described, as well as a symplas-:mic mass. Subsequently several workers (d'Arrigo, 1900; Spengler, 1905, and others) described granular forms of this organism, the work culminating in the studies of Much (1907) which received more attention than any of those previously published. This was followed/

followed by the claims of a number of French workers (Valtis, 1924, 1926, 1927; Arloing and Dufourt, 1925; Calmette _et al_. 1925; Durand and Charchanski, 1925) to have transmitted a form of tuberculosis by means of filtrates of infective material. It was claimed that the tubercle bacillus could exist in a form resembling a filterable virus, which was capable of reproducing typical acid-fast bacilli when introduced into the animal body.

These findings were viewed with considerable distrust by the majority of bacteriologists, and it is true that in some cases conclusions were drawn from inadequate evidence, but some degree of verification can be claimed in the symplasmic forms recorded by Mallassez and Vignal, Sweany, Maher and others; while Kahn described the fragmentation of the bacilli, first into micrococci, presumably equivalent to the granules of the earlier workers, and further into "dust-like particles" which returned in time to the bacillary form. Sweany describes the regeneration of bacilli from filtrates in artificial culture.

The second of the phenomena referred to above, is that of the G forms, or filterable gonidial phase, originally described by Hadley, Delves and Klimek (1931) and confirmed by Hoffstadt and Youmans (1932), Duff (1937)/

(1937), Haddow (1938) and others. These G forms are peculiar in their apparent ability to reproduce them-:selves indefinitely in that phase, forming minute colonies on solid medium. Although they are claimed to be capable of reverting to the original phase they do not by any means always do so. Hadley found that G forms could most easily be obtained from the edges of well-grown rough colonies, and accordingly formu-:lated the theory that this latter phase represented the mature reproductive stage, while the smooth form was purely vegetative. It is, however, true that the rough form is no less vegetative, and in most cases is probably more so.

The theories of Almquist and Hadley may be to some extent correlated. The former upheld the view that the enteric organisms passed through two phases, a parasitic and a saprophytic reproductive form, while the latter states that he considers the less parasitic rough variant to be the mature sexual phase. Both have been insistent on the existence of minute bacterial gonidia, which have also been described by numerous other workers.

The alternation of an immobile, filamentous form with a unicellular, flagellated stage, is very charac-:teristic of several classes of algae and fungi.

This/

This is particularly typical of the Phycomycetes, the aquatic forms of which group probably correspond to the Mycophyceae or "colourless algae" to which the bacteria were originally assigned by Cohn (see Strasburger, Engler and Prantl). These fungi also possess a sexual cycle, wherein gametes are produced, always by the filamentous form, which usually consists of non-septate, branched filaments provided with a small number of nuclei. The motile cells or "zoospores" resemble certain bacteria in the possession of flagella, though these are few in number and polar in situation. Like the filamentous stage they are distinctly nucleated. It is thus exceedingly interesting to observe that in this group of fungi alternation occurs, between a filamentous, reproductive form and a motile, unicellular, asexual form. The question arises whether the rough and smooth phases of the bacteria may have a similar significance. The structural differences, however, between these fungi and the bacteria are so great that true homology can hardly be suggested. However, by correlating the various facts and theories mentioned above, an attempt might be made to explain the changes in morphology which occur in the bacteria, by crediting them with a similar life-cycle, in which the unicellular form is parasitic/

parasitic and the filamentous form saprophytic. This would accord reasonably closely with what is known of the facts, at least as far as they relate to bacteria which are actively pathogenic in the smooth phase. It might also be applicable to those forms, usually described as "commensal", which inhabit the surfaces of the animal body, and from which the majority of pathogenic forms may possibly have been evolved. Even the fact that the S $\rightarrow$ R transformation frequently appears to be a gradual process, is not incompatible with the idea of a change from a parasitic to a sapro-:phytic form. There are, however, two problems which cannot be explained by this postulate. The first of these is the virulence of the "rough" anthrax bacillus, and the second is the fact that morphological and antigenic variation (and with the latter we must include the factor of virulence) are not always com-:pletely associated, even in those species where correlation is usually close. There even appear to be certain forms in which the major morphological variation is almost completely independent of the changes in the chemical constitution of its surface material. The streptococci and pneumococci may be isolated from pathological conditions in both the long-chained and the short-chained phase, while a similar/

similar state of affairs appears to occur in the case of H. influenzae. This latter organism, although usually isolated from lesions of the respiratory tract as a very small cocco-bacillus, when found in the nasal passages of healthy persons, or in cases of meningitis, tends to adopt a filamentous form (Dible, 1924; Scott, 1929; Smith, 1931; Pittman, 1931). As this change in morphology is associated with a change in colonial appearance, we may regard it as an S $\rightarrow$ R variation. Thus two exceedingly parasitic species, noted for their exacting requirements of growth, adopt both smooth and rough phases under conditions of parasitism.

There is yet another aspect of this question which must be considered. Among the Bacillaceae there are several species which, although normally saprophytic, occasionally occur in pathological conditions (Bain-:bridge, 1903; Grierson, 1928; Brooks, 1930). As judged by reference to described appearances, some of these appear to exist saprophytically in either rough or smooth phase (Soule; McFarland, 1898) and members of the genus Clostridium appear to behave similarly (Hoogerheide). It is to the former group that B. anthracis is closely related, and not, as in the case of the majority of pathogenic bacteria, to parasitic commensal species.

it/

It is not impossible that the large, sporing bacilli are actually the most primitive of bacteria, from which stock the simpler forms have evolved. The mechanism of evolution may have been connected mainly with the degradations commonly found in such modes of life as are habitual throughout the group. Under these conditions, as suggested by Knight (1936), the provision, ready formed of the nutrient substances required by the bacteria, has led to a progressive loss of synthetic power; and presumably, the unvarying conditions of life have led, as in many other cases, to the elimination of structures whose function has ceased to be important.

What the true significance of this alternation of phases may be to the parasitic bacteria is difficult to understand in the present state of our knowledge. It must be concluded that a variation which occurs throughout so many groups of the bacterial kingdom is of fundamental importance, but its full interpretation from the biological standpoint must await further study. The present work may serve to indicate some possible aspects of the bearing of such variation on the fundamental biology of bacteria, and its relation-:ship to the evolution of pathogenic species.

SUMMARY AND CONCLUSIONS.

(1) The structure of a morphologically "rough" colony of a bacillus or vibrio is essentially similar to that of the "Medusa head" colony of the anthrax bacillus. The bacteria are closely attached end to end in the form of threads.

(2) The bacteria composing a smooth colony tend to be separate from one another and show no characteristic arrangement.

(3) Between these extremes are a number of intermediate forms.

(4) These variations are paralleled by similar forms found among the streptococci and pneumococci.

(5) The structure of a colony depends ultimately upon physical factors and varies mainly with the degree of attachment of the component organisms.

(6) The division of the filamentous forms composing rough colonies is much less regular in occurrence and position than is that of the individual bacteria composing smooth colonies.

(7) The usually described S $\rightarrow$ R variation appears to consist of two separate changes, one affecting the morphology/

morphology of the organism, the other connected solely with the nature of its capsular material or other antigenic constituents. These may occur separately, though a considerable degree of relationship appears to exist.

(8) The biological significance of the morphological variations are discussed.

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